ANALYSIS OF RED AND YELLOW PIGMENTS IN TWO MUTANTS OF THE SIAMESE FIGHTING FISH, *Betta splendens*

A Thesis

Presented to

The School of Graduate Studies

Drake University

In Partial Fulfillment

of the Requirements for the Degree

Master of Arts in Physical Science

---

by

B. Katherine Royal

August, 1970

ANALYSIS OF RED AND YELLOW PIGMENTS IN TWO MUTANTS OF THE SIAMESE FIGHTING FISH, *Betta splendens*

by

B. Katherine Royal

Approved by Committee:

Chairman

Dean of the School of Graduate Studies

LIST OF TABLES

LIST OF FIGURES

FIGURE PAGE

TABLE OF CONTENTS

INTRODUCTION

Pigmentation, especially mammalian pigmentation, has long been a curiosity of man. It provides an aesthetic appeal displayed in various forms. It has been cultivated in domestic species including such common household animals as dogs, cats, birds and fish. Human pigment systems are similar to those of other vertebrates, yet for man, variations have created not aesthetic qualities but sociological problems. Biological and chemical research on pigmentary systems can provide information useful to man sociologically as well as medically, agriculturally and industrially.

Studies of color variation are important in several scientific areas. Medically, the biochemistry of pigments is one aspect of cell physiology. Colorimetric analysis of body tissues and fluids aids the physician in diagnosis and treatment by giving visible signs of changes in metabolic processes. Presently, melanin pigmentation is becoming increasingly important in cancer research.

The psychologist studies ethology related to pigmentation. In animals, color psychology is observed in protective coloration mechanisms and signals. Fish are excellent research animals for both since they display a variety of colors and color patterns. Many are also distinctive in their aggressive and spawning colorations.

The psychology of color can also supplement sociological

racial studies, hopefully allowing better understanding of the pigmentary nature of racial differences and how it originated.

Many geneticists are interested in aspects of the biochemistry and physiology of pigmentation. Inherited biochemical processes within an organism easily recognized as phenotypic expressions in successive generations, provide data related to factors found in the genetic make-up of the animal.

The mammalian pigmentation geneticist has aided others in the fields of biochemistry, cytology, embryology, behavior, population biology and evolution by studying the many color phenotypes in various mammals, while concurrently, these fields have provided him information for interpretation of genetic expression related to organic biosynthesis, genetic expression of genes and their products both intra- and intercellularly, genotype-environment interaction of both allelic and nonallelic genes, and finally, relationships between genetics and evolutionary biology (Foster, 1965).

Fish are becoming more important as research animals in such areas as ecology, drug abuse, behavioral studies and genetics. Used in this capacity, small aquarium fish are relatively easy to care for, they are rather inexpensive to feed and easy to house. They produce many progeny in any one mating and phenotypic variations are usually easily detected.

Early pigment research was rather general and in some cases inaccurate. More work needs to be done if correct genetic in-

terpretations are to be made from pigmentation data.

Dr. Gene A. Lucas of Drake University is currently investigating the genetics of Betta splendens (Regan). One area of interest is the relationship of genetics to pigmentation. Evidence has been accumulated for the existance of several new mutants with genetic differences displayed in color variations. Lucas proposes that mutant varieties arise because of a block in the biochemical pathway related to pigment formation resulting in a variety of color expressions. If there is a break in the sequence of pigment production, the next step cannot be made.

Other researchers do not present this viewpoint. Goodrich, Hill and Arrick (1941) list different pigments for two mutants of the Betta (common name of the Betta splendens). They view mutant varieties as expressions of different pigments rather than a block in one type of pigment formation or possibly even a qualitative reduction in one pigment allowing expression of another pigment formerly not observable.

A better understanding of fish pigment genetics would be possible if the color variations were better understood. If chemical or physical changes that result in mutant forms can be determined, then the genetic break or change can better be explained.

Pigments may be studied in terms of physiology, biochemistry and genetics or by their chemical or physical properties. The most revealing would be a combination of these. This is a com-

bined chemical and physical analysis of two mutant forms of Bettas in an attempt to identify which types of pigments are present in red and yellow mutants.

Pigmentation studies can and will lead to a better understanding of biological processes, their malfunctions and treatment. Genetic mutations, the developmental and adaptive features of the mutant strains, and possibly even sociological acceptance of phenotypic variations of Homo sapiens may be accomplished as more information is accumulated and correlated to this species as related to various research animals.

The problem selected for this study concerns color variations of red pigment in two mutants of Bettas. In their report of gene controlled pigments in fish, Goodrich et al. (1941) stated that in Betta splendens "the yellow was lutein and the red erythropterin. A deep red variety and a yellow bodied type known to fanciers as the red betta and Betta cambodia, respectively, were utilized." Fox (1953) attributed red pigment in several common aquarium fish to melanin and carotenoids.

In Drosophila melanogaster (fruit fly) there is a yellow precursor to red pterin pigment in the eyes (Fox and Vevers, 1960; Ziegler, 1961). This same sequence may be occurring in Bettas. "Yellow" and "black" mutants could result from qualitative or quantitative changes in red pigment synthesis. This study attempted to confirm the presence of lutein (yellow) and

erythropterin (red) in Bettas as reported by Goodrich, et al. (1941).

Red and yellow phenotypes were analyzed to reveal which pigments they possess. Each could possess both carotenoid and pterin pigments, or either could lack one or both types. Specifically, it was my hypothesis that the yellow phenotype is a mutant resulting from improper or inadequate red pterin synthesis.

The assumption was made that the red mutant merely represents a quantitative increase in pigment. A qualitative difference was predicted for the yellow. The presence of lutein only would suggest suppression of pterin synthesis. If pterin(s) were detected in the yellows, then the hypothesis of a qualitative change is supported.

LITERATURE REVIEW

Animal Coloration. Functionally, pigmentation may be important for several reasons. Animal vision is dependent on the light-absorbing or light-reflecting properties of rhodopsin and iodopsin which regulate the amount of light entering the eye. Reflecting pigments increase eye sensitivity and result in "eye-shine" of nocturnal animals who can see well in weak, night-time illumination.

Temperature of cold-blooded species may be controlled by light-absorbing or reflecting pigments in the skin. The dark skin pigment, melanin, serves to filter out harmful ultraviolet

solar radiation. In many animals it darkens in bright light and becomes bleached in darkness. Many colorless or transparent animals are found in darkness, especially in aquatic situations where the water serves as a protective filter.

As camouflage, colors blend with their background. These effects are caused by color patterns in animals which can alter their appearance, or by chromatophore changes regulated by the nervous system and, in some cases, the endocrine system. Amphibians, reptiles and fishes are vertebrates with this type of functional coloration.

Besides protective advantages of coloration, respiratory and metabolic functions of pigments are equally important. Colored iron and copper complexes transport respiratory reactants and products, while melanin and guanine are metabolic products.

Sexual differences in animal pigmentation may vary widely. In most instances the male shows a high degree of ornateness. Many times, especially with birds and fishes, the sexual differences in coloration are heightened during the mating or spawning season and may diminish or disappear during periods of sexual inactivity.

Studies of functional pigmentation must consider two factors: (1) what value is the color to the animal in terms of physical appearances, and (2) of what value is the pigmented substance to the animal and how are substances that happen to be colored used.

The basis of animal coloration may be chemical or physical

or some combination of the two. According to Fox and Vevers (1960),

> "The colours of an animal, like those of any object, are due to some but not all of the components of incident white light being reflected. The colour of the light reflected is said to be complementary to that which is not reflected. The removal of colours from the incident white light occurs in one of two ways; it is due either to the physical nature of the surface or to its chemical composition -- or to both. In the case of animals we speak in the first instance of structural colours, in the second of pigmentary colours."

Chemically, natural pigments, or biochromes, are colored molecules which reflect and/or transmit visible light. Biochromes are generally unaltered by physical methods such as grinding or crushing. Being molecular in nature, they express themselves independently of any structural units.

Schemochromes, on the other hand, are the colorless structural units which act to break up incident light into its component colors. They may be submicroscopic striations, facets or ridges which are altered or destroyed by physical or mechanical operations. Being mechanical in nature, they are greatly enhanced by the presence of either an underlying pigment or one superimposed on or in the structural coloring unit.

The basis of structural coloration was shown by Isaac Newton. When white light is refracted by a prism, a spectrum of colors can be cast onto a white screen. Refraction of the colored rays is inversely proportional to the wavelength, the shortest violet wavelengths being most refracted, the longer red wavelengths the least

refracted.

Wavelengths of the spectrum range from 400 mμ for the violet to 750 mμ for the extreme red. Ultraviolet radiation falls just beyond the violet end and infrared just beyond the visible reds. Both these radiation regions are invisible to the human eye, although some persons who have had their crystalline lens removed can visualize into the near ultraviolet region, and to them the mercury emission maxium at 360 mμ appears bluish (Fox and Vevers, 1960). Purple does not appear as a spectral color. It is produced as a mixture of the extremes, red and violet.

Thus the white light we normally observe contains all the spectral colors. As this light falls on any surface it is either reflected, absorbed, or transmitted in varying degrees depending on the color and quality of the surface involved. If light falls on a black surface very few, if any, rays are reflected and absorption is almost total giving the impression of black. A totally reflecting (or nearly so) surface would allow the observer to perceive white light. Various intermediate stages produce the colored impressions we perceive. For instance, if white light falls on a yellow surface, the yellow is reflected and all other wavelengths are absorbed giving the viewer the visual impression of yellow.

In animals, two types of coloration may work together. Interference and diffraction may act to produce a structural

coloration. Or a pigment ground color superimposed by a structural unit produces Tyndall coloration. Such is the case of greens in fishes, amphibians and reptiles. Light passing through yellow xanthophore pigment cells strikes crystalline guanine in guanophores just beneath the xanthophores. These scatter the incident light, which, when viewed through the yellow filter, produce the observable Tyndall green color. Other long-wave light, such as red and yellow, passes through the xanthophore filter and is absorbed by the dark melanophore background and is prevented from being reflected thus not interfering with the structural green color (Brooks, 1955; Fox and Vevers, 1960).

Nature of Pigments. The second cause of animal coloration is pigmentation. Biologists consider pigments to be colored chemical substances found in animals or plants. "Pigment" as used for paints is a substance in the form of a solid or a suspension, but not a solution. Biological pigments may also be suspensions or colloids, but some are solutions, such as carotenoids in fats, anthocyanins in plant cell sap and urochrome in urine (Fox and Vevers, 1960).

Pigmentation is a molecular phenomenon. Most compounds do not produce spectrally pure colors. They generally include all of the visible light except the absorbed fraction, the observed color of a substance depending upon the dominant or maximum wavelength transmitted.

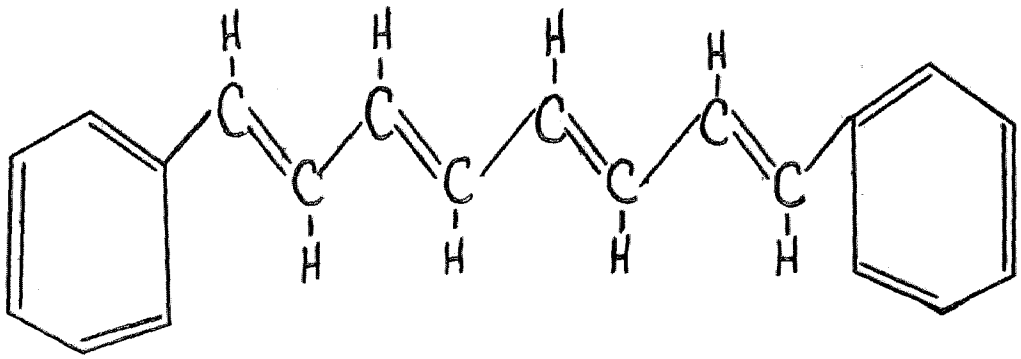
Absorption of various wavelengths depends upon the degree of unsaturation in the compound. The color-carrying groups in these carbon contining molecules are unsaturated linkages where differences in electronic energy levels are small enough to be affected by visible light. If the chromophore absorbs the shorter wavelengths, ie., those of the blue and violet region, the compound will appear yellow. Absorbed light in the blue and green regions produces red compounds, while blue or green compounds result from absorption of red or orange wavelengths. A black compound results from equal and complete absorption of all wavelengths. Molecular absorption of this type results in characteristic ultraviolet and visible spectra where the important chemical groups for color production are those containing C=C, C=O, C=N and N=N bonds (Morrison and Boyd, 1967; Willard, Merritt and Dean, 1965).

Some radicals cause hypsochromic shifts, that is shifts toward absorption of shorter wavelengths. An increased number of double bonds causes color variations particularly if the bonds are conjugated. The diphenyl polyenes with the general formula $C_6H_5 \cdot (HC:CH)_n \cdot C_6H_5$ are colored when n is large. As n, the number of HC:CH groups, decreases, the compounds are green then yellow. When n is small the compound is colorless. When a methylene group is added conjugation is destroyed and color is lost. In animal pigments, the carotenoids clearly show the dependence of color on the number of conjugated double bonds.

β-Carotene, a yellow pigment with eleven conjugated carbon-carbon double bonds absorbs in the violet region resulting in its yellow color (Silverstein and Bassler, 1963; Morrison and Boyd, 1967; Fox and Vevers, 1960). Figure 1 illustrates this effect in carotenoids.

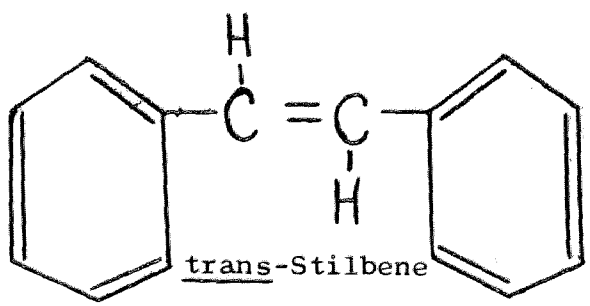
While infrared absorption and the resulting spectrum is a phenomenon characteristic of molecular stretching and bending vibrations, ultraviolet and visible absorption result from transitions between electronic energy levels. Electronic transitions can occur with σ electrons, π electrons or n electrons (non-bonding electron; one of an unshared pair). The electrons are excited to higher energy levels with the absorption of ultraviolet energy. The excited state exists for a finite length of time. During this time relatively small amounts of energy may be transfered. In returning to the ground state, radiation of a different wavelength than that absorbed is emitted; this is fluorescence (Willard, et al., 1965).

If the absorption band or bands are in the ultraviolet region the substance will be colorless to the eye. However, the presence of certain unsaturated groups in the compound cause the wavelength of absorption to shift, such that transmitted wavelengths appear in the visible regions. When this happens, the compound appears to be colored. Although compounds containing the radicals $-NH_2$, $-OCH_3$, -Cl and -OH are normally colorless, these same radicals cause bathochromic shifts, that



Diphenyl Polyene: n = 4

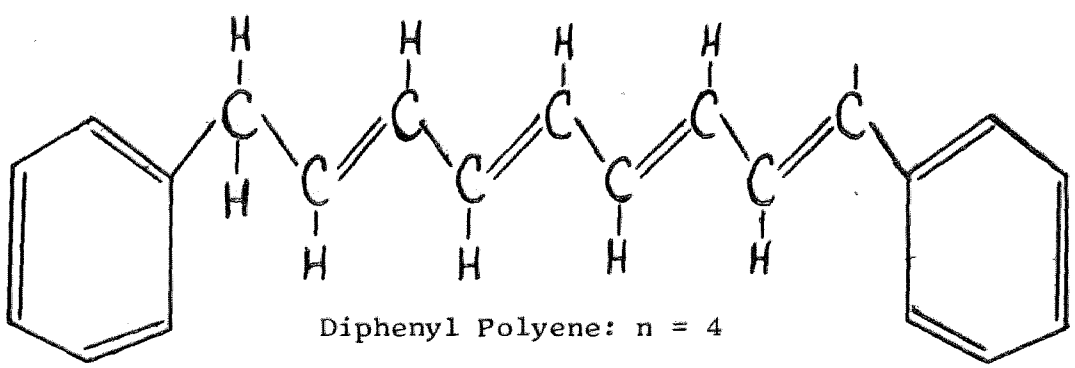
Yellow-green, with blue-violet absorption



trans-Stilbene

Diphenyl Polyene: n = 1

Colorless, with ultraviolet absorption

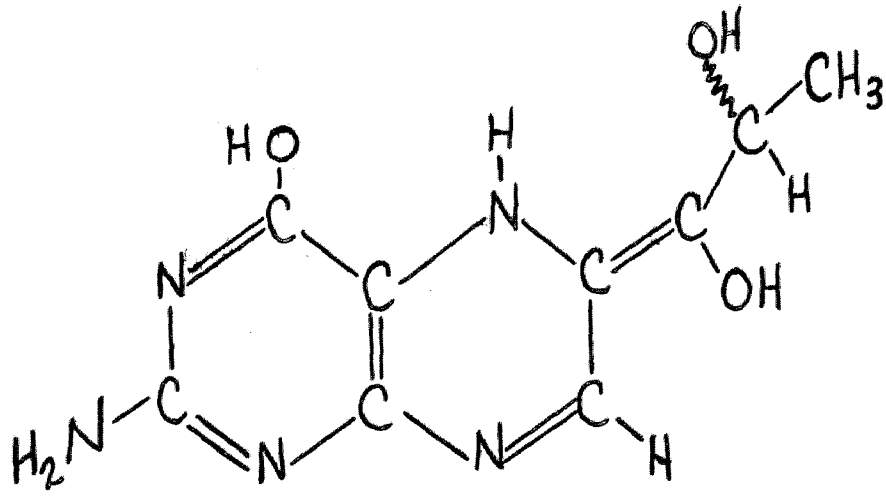


Diphenyl Polyene: n = 4

$-CH_2$ group added

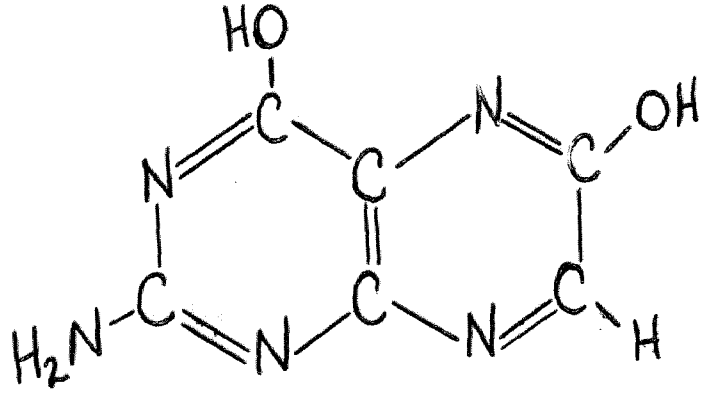
Colorless, with ultraviolet absorption

Figure 1. Hypsochromic shift displayed in carotenoids.



DROSOPTERINS

Orange, with blue-green absorption



XANTHOPTERIN

Green-blue, with red-orange absorption

Figure 2. Bathochromic shift displayed in pterins.

is, absorption of longer wavelenghts. Benzene normally absorbs in the ultraviolet region, but addition of one of many radicals causes bathochromic shifts to the visible region. Figure 2 illustrates a bathochromic shift in the fluorescing pterin pigments when a -OH group is substituted for a complex conjugated carbon chain (Fox and Vevers, 1960; Willard, et al., 1965).

Color and chemical reactivity are parallel expressions of molecular unsaturation, therefore research on pigmentary compounds will increase understanding of metabolic activity.

Betta Pigments. Two mutants of the Siamese Fighting fish, Betta splendens, were selected for this pigment research. Through selective breeding vivid colors have been developed in these fish. Domestic fish are so varied in color and form that it is difficult to imagine the long-finned, colorful fighters as decendents of the original short-finned, rather "colorless" wild-type Bettas pictured in Figure 3. G.A. Lucas (1968) defined a standard color of wild-type Bettas for use in comparison of various pigment mutants:

> "The body color is light tan to deep brown depending upon physiological state, the color of internal tissues, and superficial pigment cells, specifically melanophores (brown-black color), xanthophores (yellow color), and limited amounts of green metallic color produced by reflection of light through granules or crystals of guanine in "iridocytes." Erythrophores (red color) are found in limited areas of the head and fins."

Domestic varieties may include intensified reds and blues, lavenders, iridescent green, cornflower-blue, blue and white

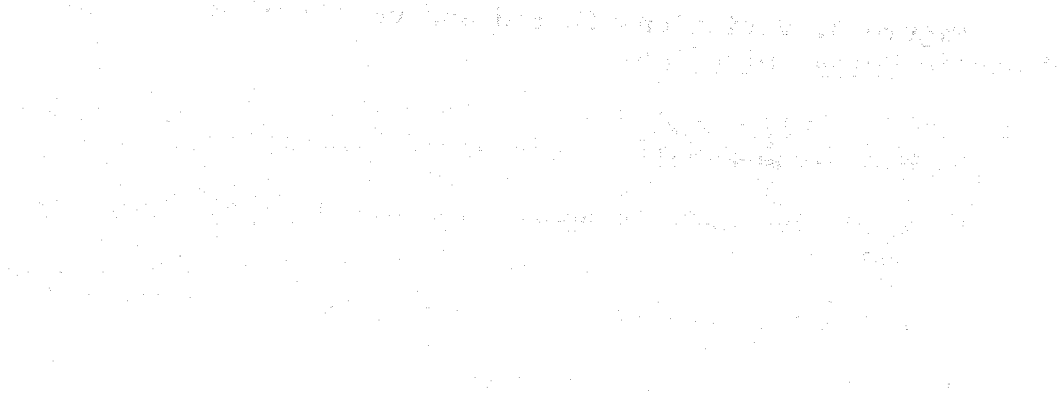


Figure 3. Variations in red and yellow pigmentation of domestic Betta splendens.

a. Wild-type female. Note areas of red coloration.

b. Wild-type male in aggressive display showing darker body.

c. Red male. Red is extended to cover the entire body.

d. "Pale yellow" non-red male with limited iridocyte color.

e. A "yellow" cambodian Betta.

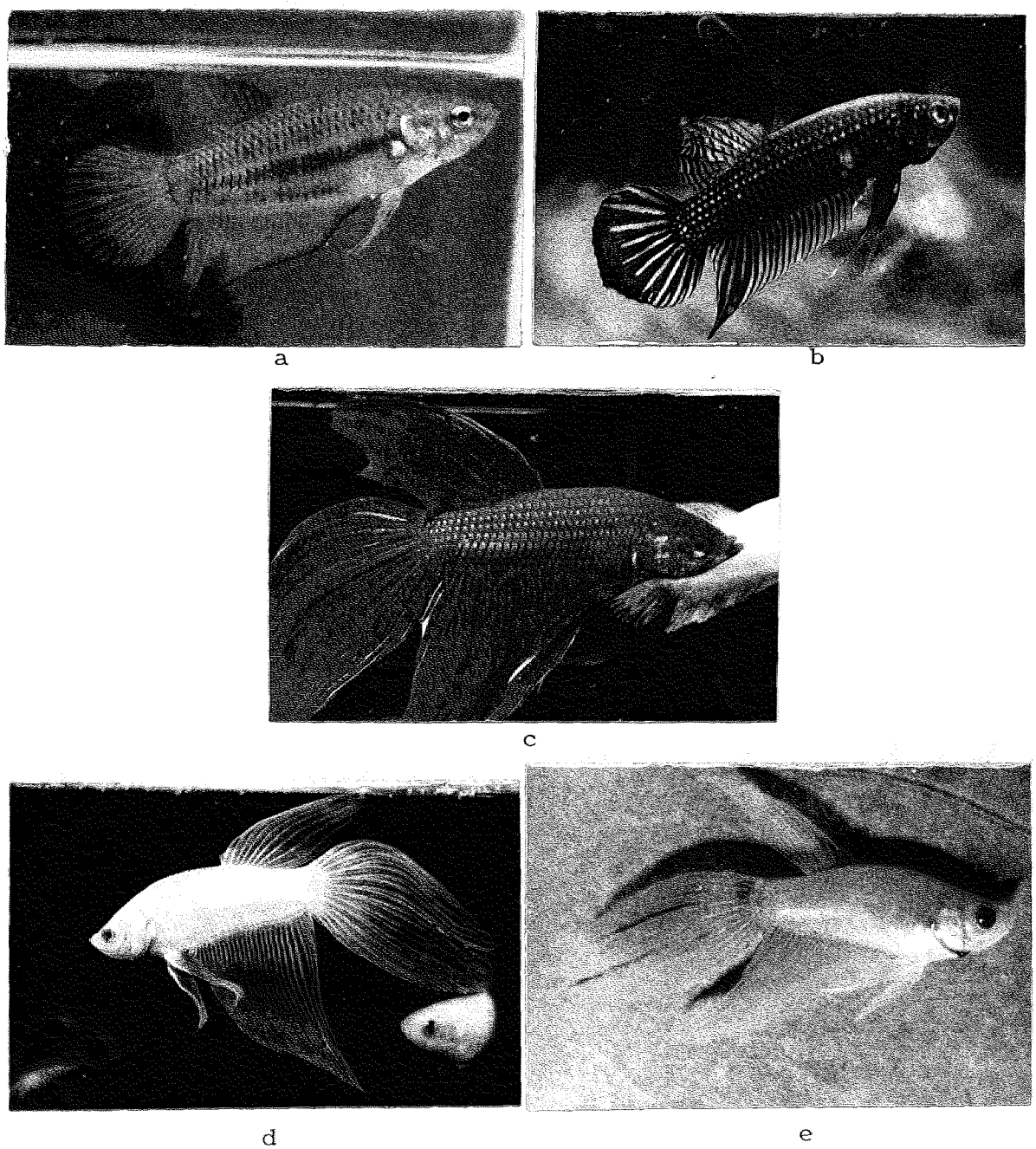


Figure 3. Variations in red and yellow pigmentation of domestic Betta splendens.

("butterflies" or "variegated"), yellowish and reddish creams with bright red fins ("cambodias"), and black (melanistic). Variations in black ("Black Lace") and yellow ("white" and "pastel") are currently being developed.

The origin of pigments in fish is variable. Some biologists believe that fish, as well as other animals, assume the color of the particular plant on which they live merely because they feed on that plant. The plant pigment is either directly obtained by feeding on the plant or indirectly consumed by eating smaller herbivorous animals. For carotenoid pigments this would seem to be the case since animals are unable to synthesize their own even though they are among the most widespread of animal pigments (Fox and Vevers, 1960).

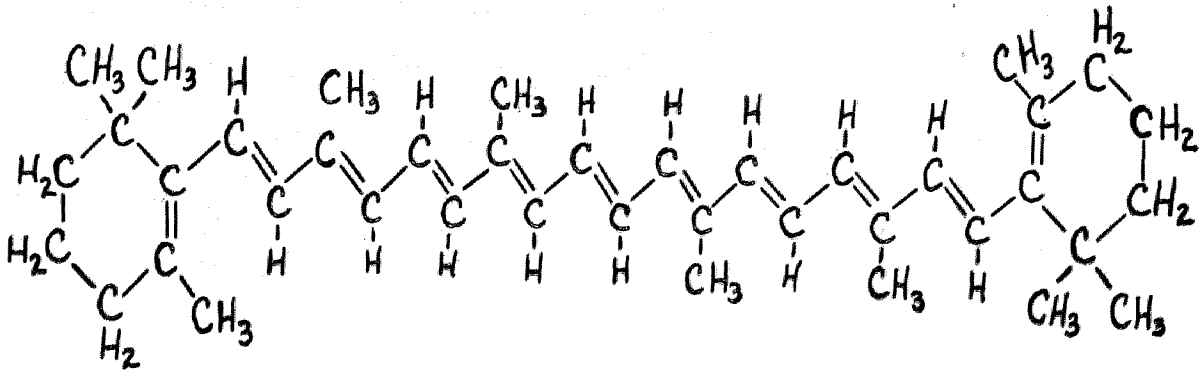
Other biologists feel this is not true. Changes in coloration can be effected experimentally, therefore there is no dietary dependence. Color matching, such as bottom mimicry in flatfishes, is effected somehow through the sense of sight. Fox (1953) partially explains this discrepency by pointing out that photic factors have been shown to influence melanin, guanine and xanthophylls. He agrees with Sumner that such biochromatic variations serve the animal as camouflage, but the actual biochemical and physiological paths and processes involved are still unknown. It seems that the origin of pigments depends upon the kind of pigment considered.

Goodrich, Hill and Arrick (1941) propose that Betta colors

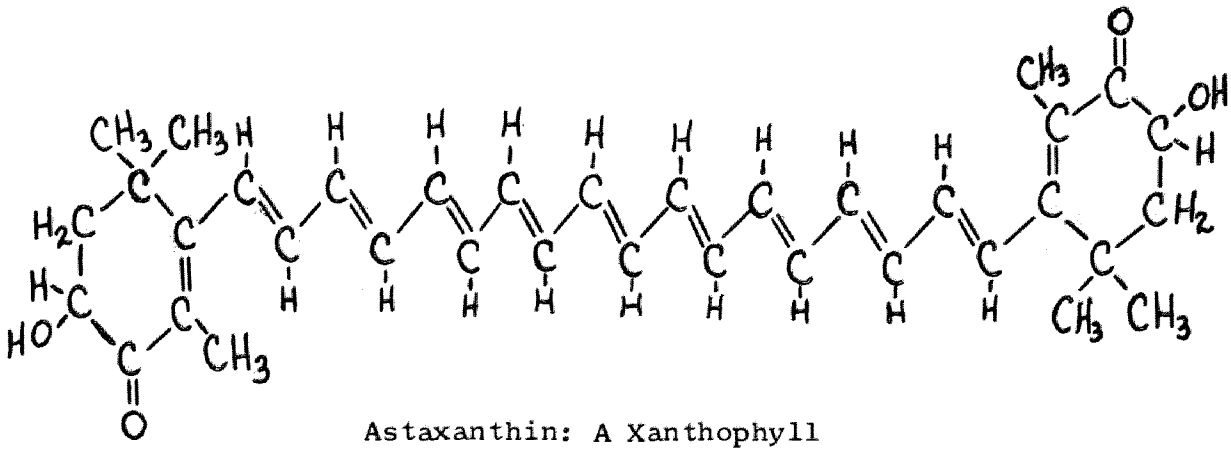
are produced by yellow lutein, red erythropterin and black melanin. Goodrich and Mercer (1934) suggest that light scattered by small hexagonal crystals of guanine produce steel blue, blue or green when this physical effect is combined with background pigment. Cells containing these crystals are termed iridocytes or guanophores, while melanin is contained in melanophores. The carotenoids and pterins are the pigment classes of direct concern in this research, therefore a more detailed discussion of these follows.

Carotenoids are the most universally distrubuted pigments in the plant and animal world. They are generally red, orange or yellow with the best known of the family being carotene.

Important to carotenoids are the lipids. As defined by Bloor (Cantarow and Schepartz, 1963), lipids are "a group of naturally occurring substances consisting of the higher fatty acids, their naturally occurring compounds, and substances found naturally in chemical association with them." Lipoproteins are compounds of lipids attached to proteins. Carotenoid pigments are lipid-soluble and are often found associated with lipids in nature. It is the dissolved carotenoid pigments (or other chromolipids) in fats that produce the yellow color since fats themselves are generally white. Before their similarity to plant carotenoids was determined, animal carotenoids were termed lipochromes since they were found dissolved in fats (Fox and Vevers, 1960).



β-Carotene: A Carotene



Astaxanthin: A Xanthophyll

ASTAXANTHIN: A red carotenoprotein. In ketol form it is red, in enol form (and absence of air), it forms a deep blue alkali salt. It is unique in that it gives single-banded spectrum with maximum in petroleum ether at 472 mμ.

Figure 4. Typical members of the two carotenoid classes (Fox and Vevers, 1960).

As related to animal colors, carotenoproteins (carotenoids united with proteins), are significant for two reasons: first, they make the pigment water soluble; second, they change the color of the compound. Besides the red, orange and yellows already mentioned, carotenoproteins may be purple, blue, grey, brown or almost black.

Pigment concentration also affects the observable color of the yellow-orange-red effect. It is the light transparent yellow, sparsely dissolved in fat droplets that provides the yellow filter for Tyndall scattering and the resulting structural green.

Carotenoids are non-nitrogenous compounds generally divided into two groups: (1) "carotenes," the hydrocarbons, and (2) "xanthophylls," the oxygen containing derivatives. "Lutein" has been used synonymously with xanthophyll, although lutein is only one of several xanthophylls. Figure 4 gives structural diagrams for each of these carotenoid classes.

β-Carotene is a typical hydrocarbon carotene characterized by a chain of alternating double and single bonds with ionone rings at each end of the chain. Because its structure is well known and numbered, β-carotene is the most important carotenoid for analytical purposes. Figure 5 gives its structure together with the now accepted numbering system.

> "In a molecule containing only one β-ionone residue, the ticked numerals are allocated to the half of the molecule not containing this residue. In almost all the naturally-occurring C_{40} carotenoids, the central chain (from carbons 7 to 7') is the same as in β-carotene, variations occurring only in positions 1-6 and 1'-6'." (Goodwin, 1955)

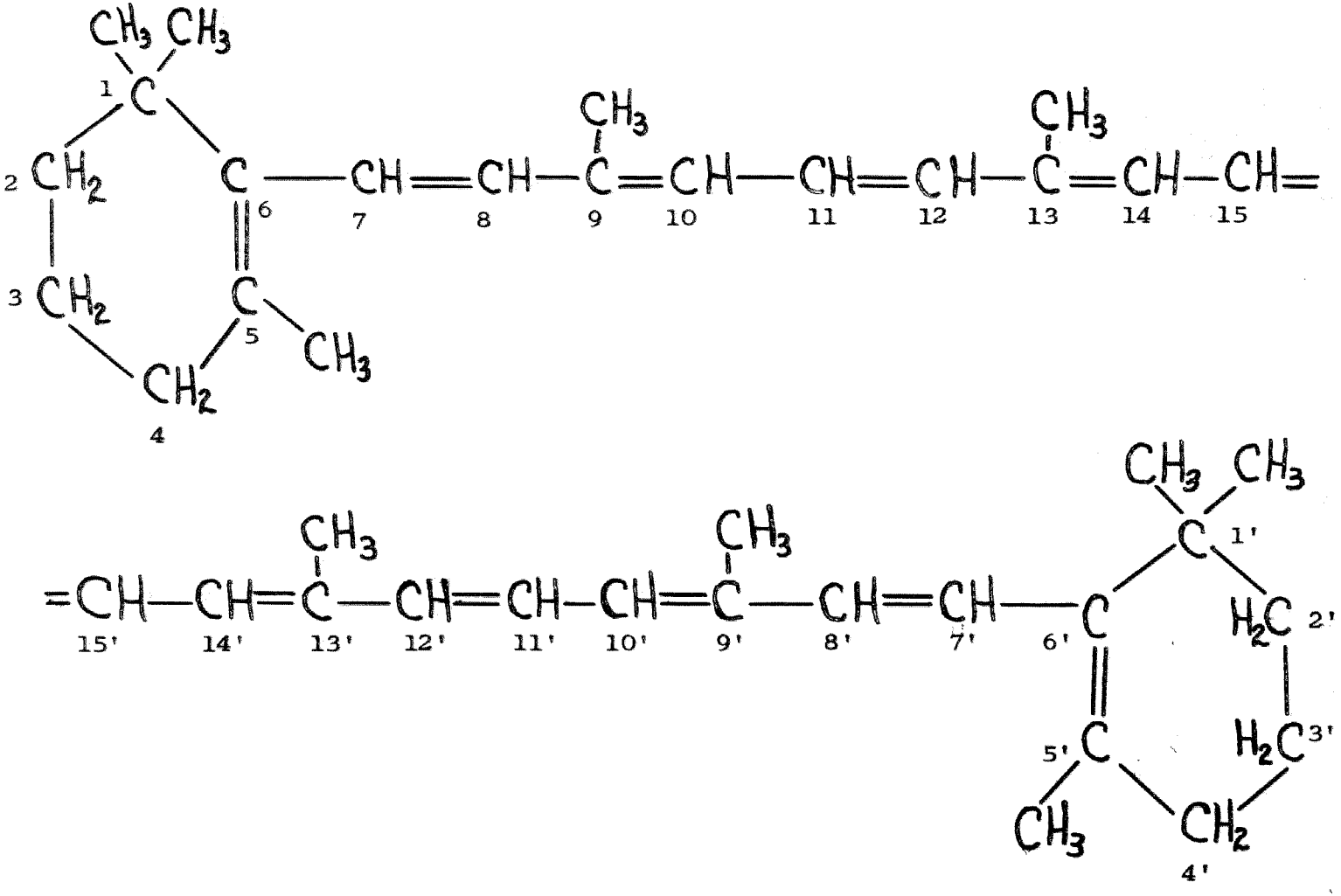


Figure 5. β-Carotene: Carotenoid system of carbon numbering. (Goodwin, 1955)

Both carotenoid classes are characterized by isoprene residues, generally 8, resulting in a C_{40} chain structure. Two lateral methyl groups are in the middle of the molecule and occupy the relative 1,6 positions, in all other cases the relative positions are 1,5 (Goodwin, 1955).

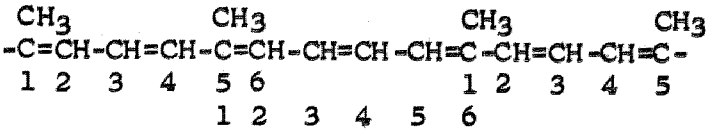


Figure 6. Center of carotenoid molecule.

The oxygen of xanthophylls can occur in hydroxy-, methoxy-, epoxy-, carboxy-, or carbonyl groupings. Hydroxy derivatives occur either in the free state or esterified with fatty acids, such as palmitic acid. Although many xanthophyll structures have not been well determined, the probable structure of lutein, cited as occurring in Bettas (Goodrich, et al., 1941), is presented in Figure 7.

Structural diagrams of carotenoids indicate the possibility for <u>cis</u> → <u>trans</u> isomerization. Investigations by Zechmeister and his workers reveal that although <u>cis</u>-isomers occur in nature, the predominant form of carotenoid pigments is the all-<u>trans</u> form (Goodwin, 1955).

Other properties of carotenoids include readiness to crystallize when finally obtained in sufficiently pure quantities, their insolubility in water, and solubility in organic solvents such as acetone, petroleum ether and chloroform. Their conjugation

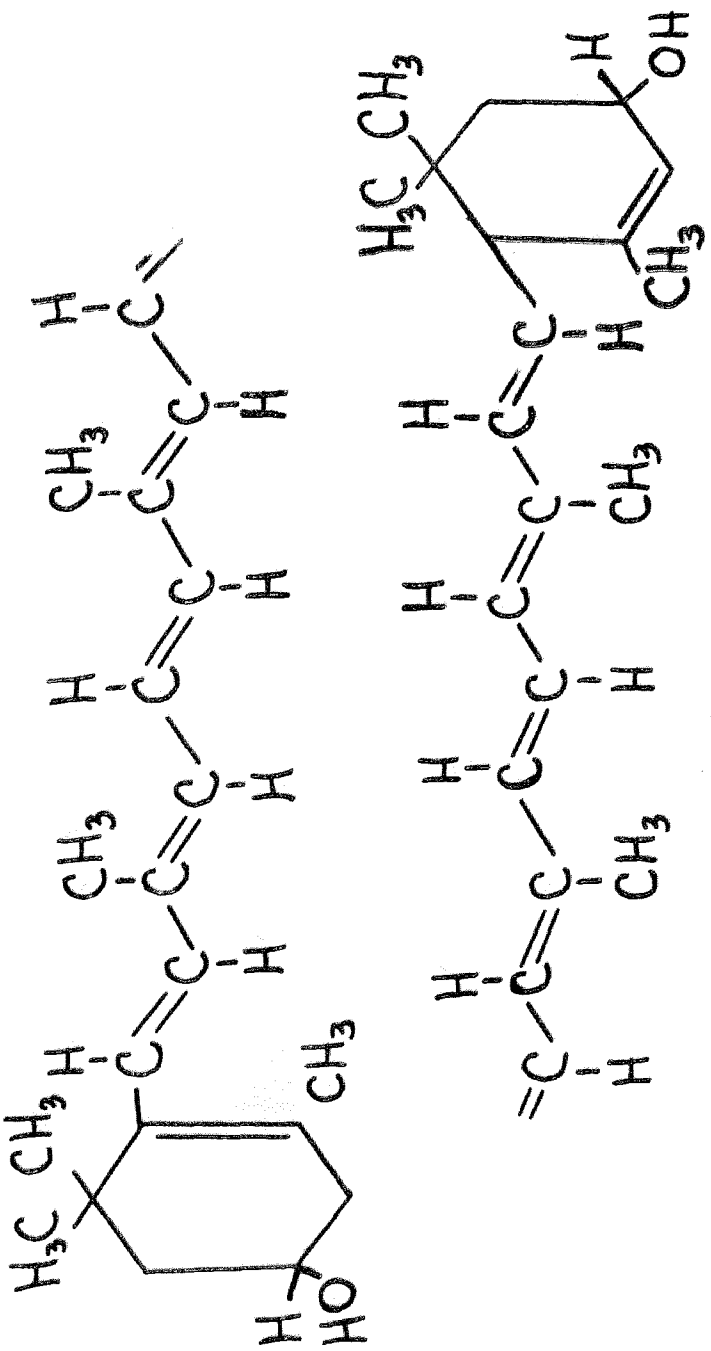


Figure 7. Lutein - A xanthophyll carotenoid.

makes them readily oxidized by atmospheric oxygen and easily bleached by light. Instability also occurs if mineral acids are present. At low oxygen pressures and low temperatures, carotenoids are remarkably stable, with 6,000 year old samples found thus preserved in marine muds.

Responsible for carotenoid colors is the conjugated system. Variations in color depend on the order, increasing number of double bonds and the presence of certain radicals. Characteristically, they display two or three absorption bands, although some display only one. Generally, these are in the blue to violet spectral region, sometimes extending into the green range. The absorption maxima depend on the solvent used with light petroleum giving the lowest values and carbon disulfide the highest.

Goodwin (1955) reports several other factors affecting absorption maxima. These include:

(1) displacement towards longer wavelengths if the ring system is opened,

(2) displacement towards shorter wavelengths if the terminal double bond is replaced by an epoxide group,

(3) displacement towards longer wavelengths if a conjugated keto group is added even though an unconjugated keto or hydroxyl group has little effect.

(4) Cis-trans isomerization profoundly affects not only the position of the absorption maxima but also the actual shape of the absorption spectra curve.

Although carotenoids are generally considered to be highly colored compounds, recent plant studies have resulted in the discovery of two partly hydrogenated carotenoids that are color-

less substances. They are becoming increasingly important in carotenoid biochemistry. Since "carotenoid" connotes color, these two colorless compounds are refered to as "colorless polyenes." Little information is available, however, phytofluene is found to fluoresce bright green in ultraviolet light, and phytoene shows an absorption spectrum in the 265-295 mμ region. These can be chemically separated on an activated alumina flowing chromatographic column, where petroleum ether elutes the phytoene before the phytofluene (Goodwin, 1955).

Since animals cannot synthesize their own carotenoids, they must get them directly or indirectly from the food they eat. Astaxanthin is an abundant carotenoid in animals, but uncommon in plants and algae. This strongly suggests animal modification of this carotenoid from plant carotneoids. Fox and Vevers (1960) describe a recent investigation of the origin of astaxanthin in brine shrimp, Artemia salina:

> "The animals were fed on the unicellular green alga Dunaliella tertiolecta, which was found to contain six carotenoids. The orange brine shrimps contained the same six pigments and also esterified astaxanthin, which was absent from the food. In addition a keto-carotenoid, which too was lacking in the alga, was present in the brine shrimps; it is probable that the keto-carotenoid is an intermediate stage in the formation of astaxanthin, as has previously been suggested in starfish."

Since brine shrimp are commonly included in the diet of Bettas, it is strange that astaxanthin was not found by Goodrich, et al. (1941).

Other carotenoids found in converted forms in animals include carotenes to xanthophylls, β-carotene and lutein to γ-carotene and astaxanthin, with esterification of the lutein. Lutein is the only carotenoid reported by Goodrich, et al. (1941) to be present in Bettas.

Fox (1953) classified animals according to their carotenoid absorption showing that alternate possibilities of treatment can occur within the animal.

(1) Carotene animals selectively assimilate and store hydrocarbon carotenoids.

(2) Xanthophyll animals store the hydroxy-carotenoids, rejecting carotenes or possibly converting them to xanthophylls.

(3) Non-carotenoid animals store little or no carotenoids; they are either voided or converted by degradation.

(4) Non-selective animals assimilate and store both carotenoid classes.

Goodwin states that carotenoids are generally found in the skin and ovaries of fish. Although richly accumulated in ovarian tissues, carotenoids rarely occur in male gonads. This difference suggests sex hormones to be one factor affecting deposition. Other factors cited include light and temperature (Fox and Vevers, 1960; Goodwin, 1964).

Fox and Vevers (1960) outline several simple chemical tests that can be used to distinguish carotenoids from pterins. They can be separated on the basis of solubility, but the mixtures resulting within the separated chemical groups are only partially

separable in this manner. For carotenoids dissolved in petroleum ether, the addition of 90% methanol:10% water will bring separation as xanthophylls collect in the methanol hypophase and the hydrocarbons, their oxides and most xanthophyll esters collect in the petroleum ether epiphase.

All carotenoids are easily soluble in solvents such as chloroform, benzene, acetone and carbon disulfide, with the hydrocarbons being soluble in light petroleum ether but almost insoluble in ethanol or methanol. The xanthophylls display opposite characteristics and become more soluble in alcohols as the number of oxygens present increases.

Once separated, the carotenoids can be tested for color reaction with concentrated sulfuric acid. When dissolved in anhydrous, ethanol-free, chloroform solution, all carotenoids give a green-blue color when treated with concentrated H_2SO_4. The color is intense but transitory (Goodwin, 1955).

Fox and Vevers (1960) showed goldfish color to be mainly carotenoid by grinding the fins in a mortar with acetone and extracting. The fin material, after repeating the procedure three or more times, becomes almost colorless, while the acetone solution becomes golden-yellow. The colored pigments in the acetone are easily separated in petroleum ether. If the colored petroleum ether solution is then exposed to sunlight, fading will occur.

Pterins were first isolated by Gowland Hopkins in the years 1889-1895. Using white and yellow butterflies, Pieris brassicae and Gonepteryx rhami, he isolated a white substance thought to be uric acid and a yellow pigment shown to be related to uric acid (Fox and Vevers, 1960).

The work of Wieland and his associates from 1925 on produced xanthopterin (yellow-xantho; a wing-pterin) and the colorless leucopterin from the wings of 215,000 white butterflies, Pieris napae. Later work produced red erythropterin causing coloration in the forewings of the orangetip, Euchloe cardamines. Formerly termed "lepidopterin" since they are encountered frequently in butterfly wings, the name of this pigment class today has been shortened to "pterin."

Because of their close chemical and metabolic relationship to purines, pterins were formerly classed with them. However, purines, including guanine and uric acid, are generally white crystals, making them difficult to class as true pigments. Pterins, on the other hand, range in color from white through yellow, orange and red.

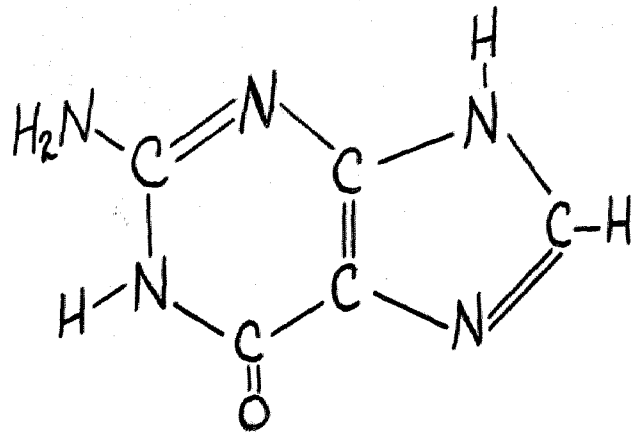
Pterins also show a close chemical and functional relationship to flavins, another family of nitrogenous, yellow, green fluorescent, water-soluble pigments. Because of their similarities, flavins should be distinguished from pterins in research. However, the problem is alleviated by the fact that flavins do not contribute to external animal coloration, therefore leaving

coloration to the pterins (Fox and Vevers, 1960). Figure 8 presents comparative structures of the three chemical classes pterins, purines and flavins.

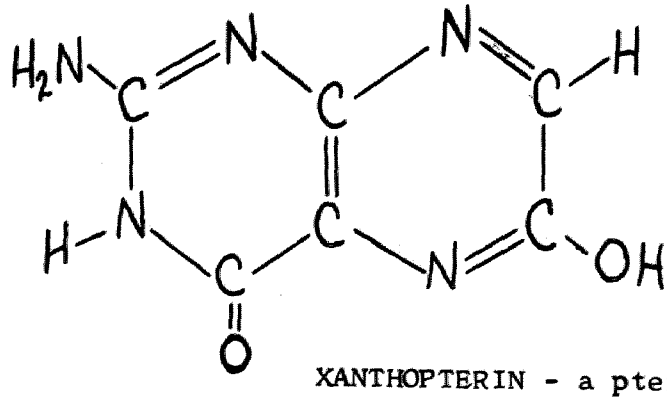
Pterins occur as integumentary pigments only in arthropods and poikilothermal vertebrates (Fox and Vevers, 1960). In other animals they occur as colored products of excretion. Pterins are also found in mammalian liver, feces and urine, hay alfalfa and other plants. The fruit fly, Drosophila melanogaster, contains a red eye pigment of the pterin class that interests geneticists, while a blue-fluorescing pterin, fluorescyanin, in the scales of many fishes biochemically resembles some of the B vitamins.

Xanthopterin and leucopterin are crystalline granules as shown by polarized light, while the faintly yellow water-soluble pterins are non-granular or crystalline. Pterins are located in tissues by their blue fluorescence. Upon addition of acetic acid or ammonia the protein-pigment bond is broken and fluorescence appears immediately. Localization must be determined at once since the fluorescing substance rapidly diffuses away (Fox and Vevers, 1960).

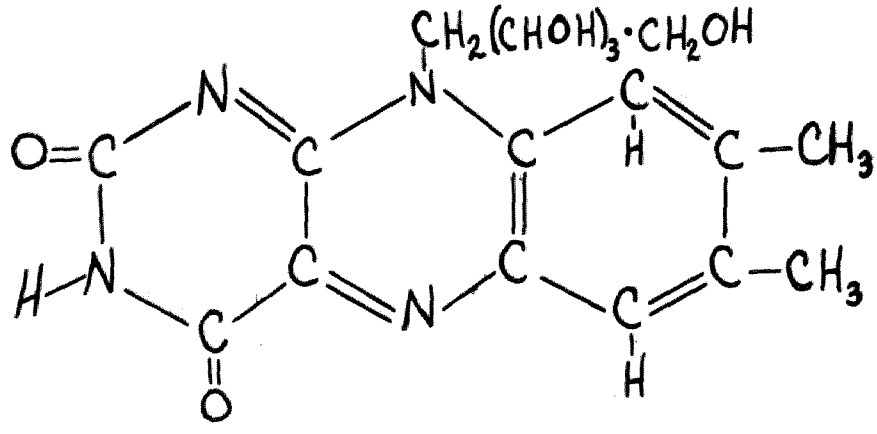
Pterins occur in combination with other pigments in chromatophores of several different animals. The lipophores of goldfish and many amphibians contain combinations of pterins and carotenoids. Such cells are cited by Goodrich, et al. (1941) and are refered to as xantho-erythrophores. Pterins are also



GUANINE - a purine



XANTHOPTERIN - a pteridine



RIBOFLAVIN - a flavin

Figure 8. Purine, pteridine and flavin structures.

found in iridocytes in yellowish cells called guanophores. In addition to pterin, they contain some guanine or hypoxanthine. Figure 9 shows several examples of various pigmented Betta scales.

Of perhaps more interest is the occurrance of pterins in melanophores. Pterins may impart a delicate yellowish, but distinct veil over the melanophore.

> "The fluorescence microscope shows that in tadpoles, and also in young fishes, the first appearance of fluorescence on the addition of ammonia is in the hyaline bubble-like outgrowths from melanophores. In these bubbles sulphur-yellow granules appear late in development and then a pterinophore separates off from the melanophore. Colourless, blue-fluorescing pterins may often be present in melanophores themselves, ... but this is not always so ...." (Fox and Vevers, 1960)

In light of this, it is possible that the redox properties of pterins assist in melanogenesis. Being an end product of metabolism, the chemically reversible oxidation steps can be followed by the colored products produced at each stage. The yellow, orange and red stages are culminated by an intensely black product if the process goes uninterrupted to completion. Figure 10 shows the known steps of melanogenesis.

Isaka (Fox, 1953) has shown that xanthopterin inhibits melanogenesis <u>in vitro</u>, while riboflavin exercises the opposite effect. The possibility of an antagonistic interaction between pteridine and iso-alloxazine rings existed, and

> "Isaka's experiments led him to conclude that the reaction, xanthopterin + $\frac{1}{2}O_2 \longrightarrow$ leucopterin + H_2O, inhibits the darkening of tyrosine-tyrosinase systems, not in the initial (red), nor even in the

> second (leuco) phase, but in the third and final stage of oxidative melanogenesis. His proposed reaction is expressed as follows: DOPA + xanthopterin + $O_2 \longrightarrow$ phenylalanine quinone + leucopterin + H_2O." (Fox, 1953)

This association with melanophores has prompted tumor growth studies as related to pterins. Fox (1953) reports that

> "Preliminary investigations by Lewisch, Laszlo, Leuchtenberger & Leuchtenberger (1944) indicate that xanthopterin serves to deter tumor growth in mice. Leucopterin not only failed to inhibit tumor development, but seemed to neutralize the effects of xanthopterins. This study should be repeated and extended."

Other medical studies show pain induction or reduction by pterin regulation, while xanthopterin has been used to cure or prevent anemia in rats, monkeys, dogs and young salmon (Fox, 1953).

Because of their association with melanin, a known metabolic pigment, and because of its relation to purine compounds, notably guanine, a constituent of the nuclear material of all cells, it is presumed that pterins may play an indispensable role in cell metabolism. Fox (1953) suggests that certain pterins occurring conjugated with proteins may, like the flavoproteins, serve as coenzymes. Pterins, unlike the flavins, are sensitive to oxidation with permanganate in acetic acid. However, like the flavins, certain pterins can be reduced to non-fluorescing leuco derivatives, a reversible effect when shaken with air. This behavior is similar to the flavins' physiologically important roll as cellular redox agents, suggesting that pterins might occupy a similar role.

Figure 9. Variations in pigment cells in Betta splendens

a. Composite color produced by black, red and yellow pigment cells in red Betta. (100x)

b. Melanophores and "lipophores" in non-red Betta. (970x)

c. Black and red cells observed in scales of red fish. (970x)

d. Absence of melanophores and presence of "lipophores" in scale from yellow phenotype. (440x)

e. Black and red pigment cells where red seems to have replaced yellow. (440x)

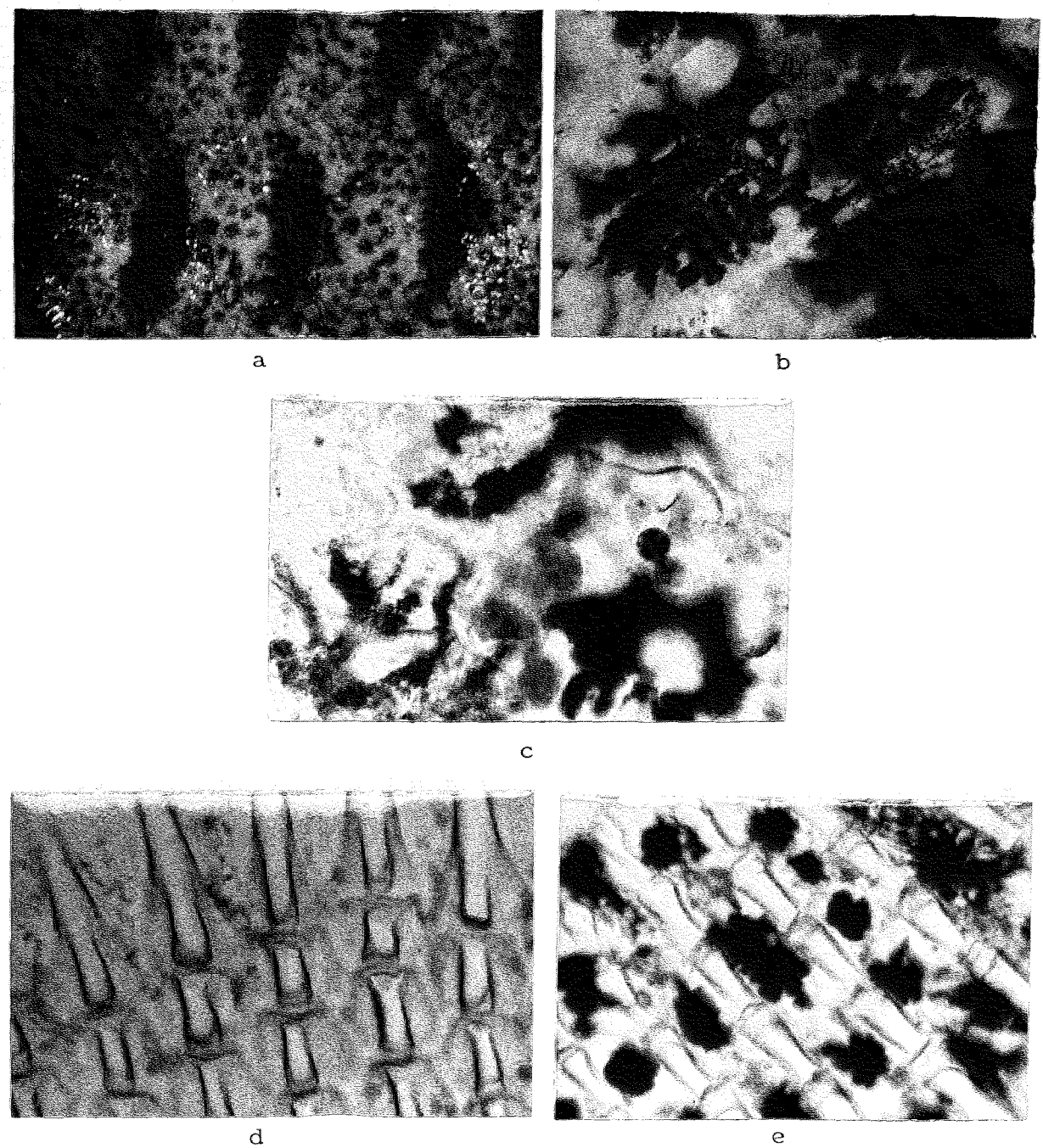


Figure 9. Variations in pigment cells in Betta splendens.

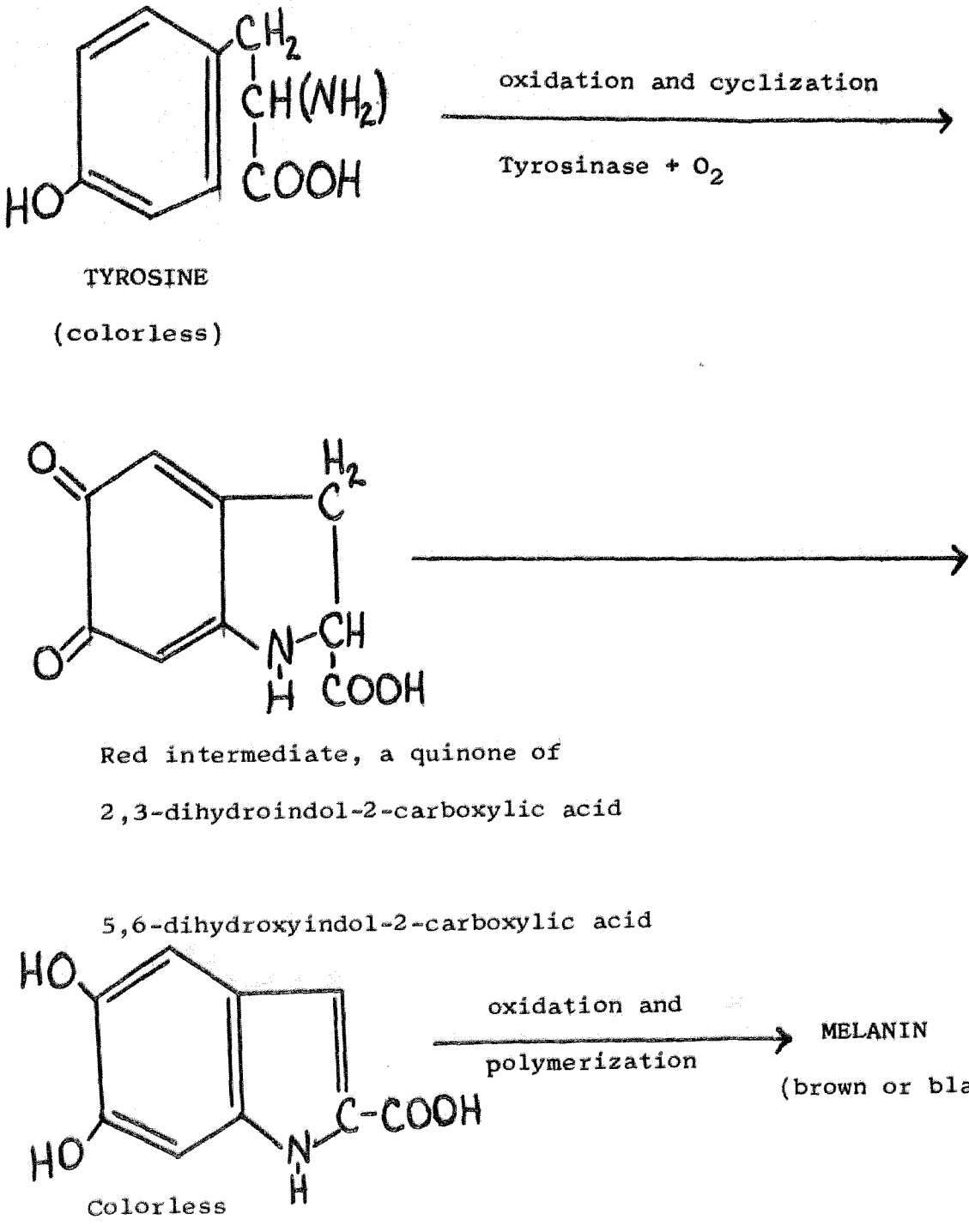


Figure 10. Tyrosine (colorless) converted to melanin pigment.

It is apparent that research in this area of pigmentation is limited, with the need for pterin information becoming increasingly important.

Pterins are heterocyclic, nitrogenous, catabolic compounds with color produced by electronic transitions within C=C, C=O, C=N and N=N groups; bathochromic effects are produced by $-NH_2$ and -OH groups. Purrmann (Fox and Vevers, 1960) has shown the pterins to have a pteridine skeleton that is heterocyclic and similar to the purine skeleton of uric acid and guanine (Figure 8). Degradation and synthesis has established the structures of xanthopterin and leucopterin, the latter containing one more oxygen atom. Reports by Fox (1953) and Hadorn (1962) indicate that pterins are generally present in minute quantities with enormous amounts of raw material needed to extract enough of the chemical for detailed structural analysis.

Since pterins contain 30-40% nitrogen they are fairly polar. Somewhat water soluble, they are insoluble in common organic solvents. This is the basis for their separation from carotenoids. They are more soluble in dilute acids or bases than in neutral aqueous media due to their amphoteric nature. Melting points are not well defined for pterins, since only a few may be obtained in crystalline form. Others are recovered only as microcrystals or crystalline derivatives of other compounds. Generally, they have a tendency to decompose at higher temperatures further hindering any melting point deter-

mination. Because of their sparse occurrance in tissues and their reluctance to crystallize in pure form, chemical identification is difficult, and diagnosis is dependent on nitrogen content, fluorescence and other absorption properties.

The accepted method of isolation is chromatography, both column and paper. Best identification is achieved by determining fluorescent and other properties (Hadorn, 1962; Fox and Vevers, 1960; Cantarow and Schepartz, 1963). Pterins may fluoresce reddish, yellowish-green or blue depending on pH and the salts present. Absorption maxima at 340-370 mμ result in fluorescence. Generally, pterins do not fluoresce in nature but do in solution after pterin-protein bonds are broken. Water-soluble pterins fluoresce blue in solution, their fluorescence disappearing if a reducing agent is added, and may reappear on shaking or standing in air, typical of their redox behavior (Fox and Vevers, 1960).

Fox, in Animal Biochromes and Structural Colours (1953), discusses the more common animal pterins; a summary of this follows:

(1) Erythropterin is red and the darkest colored member of this class. Orange colored butterfly markings bear mixtures of erythropterin and xanthopterin. It may be ammonically extracted, followed by precipitation with hydrochloric acid. It is very water soluble giving a bright, intense red color. Its nitrogen content is lower than most pterins, and it fluoresces

in 2N-acetic acid. It is more strongly adsorbed on alumina than xanthopterin.

(2) Xanthopterin may be recovered as the insoluble barium salt. In free form it gives the murexide reaction. Identification is further facilitated by its yellow to yellow-green fluorescence in weak acid, clear blue in neutral solutions and blue-green in weak alkali. It is firmly adsorbed by alumina and becomes chemically altered although it still fluoresces.

(3) Drosopterin, a darker red than erythropterin, was purified by Lederer in 1940 from the eyes of Drosophila. In powder form, it contains 42% C, 5.6% H and 19% N. It has an absorption maximum at 465 mμ in water as compared to 450, 420 and 300 mμ maxima for erythropterin in water.

(4) Leucopterin was first mistaken for uric acid. White or colorless, it is extracted with ammonium hydroxide; its precipitation and recrystallization yields minute quantities of ammonium salts. It gives the murexide reaction.

(5) Chrysopterin is an acidic pterin intermediate in properties between xanthopterin and erythropterin. Similar to erythropterin, it fluoresces violet-blue in acetic or hydrochloric acid; like xanthopterin, it is strongly adsorbed on alumina exhibiting a green-yellow fluorescence.

(6) Fluorescyanin was discussed in 1946 by Polonovski, Busnel and Pesson. Encountered in 1939 by Fontaine and Busnel in the scales of fishes, it exists naturally as a chromoprotein,

similar to riboflavin. Fluorescyanin gives typical pterin tests, and may be reversibly reduced, being more readily oxidized by atmospheric oxygen. It has been suggested that this pterin is a hydrogen transporter.

(7) Mesopterin is intermediate to leucopterin and xanthopterin showing color, solubility, chemical composition and feeble alkaline properties between these pterins.

(8) Guanopterin, as the name implies, exhibits properties similar to guanine. Fine colorless needles are produced by crystallization.

When characterizing and identifying the pterin class, confusion and possible overlap may occur as pterins are discussed from one animal class to another. For example, the red pterin of insects (Drosophila) is "drosopterin," while the red pterin of butterflies and fish is "erythropterin." Absorption maxima are valid differences given for these red pigments. However, using refined techniques, it could possibly be shown that these two red pterins are the same!

Another example arises with "ichthyopterin." Reported by Fox and Vevers (1960), "ichthyopterin" resembles "fluorescyanin," but "... the former is actually a mixture of four pterins separable by paper chromatography." Fox (1953) also associates these two pterins, but characterizes "ichthyopterin" with a chemical formula, $C_7H_8O_3N_4$, and does not suggest it to be a pterin mixture.

As outlined previously, pterin-carotenoid separations are based on solubilities. In animal tissues, pterin presence is indicated by blue and/or green fluorescence when 20% ammonium hydroxide is added. For example, white pierid wings yield a blue fluorescing substance, while yellow wings produce green. These color differences are thought to be caused by different proportions of pterins present. However, it could be that differences in fluorescence are caused by the presence of two different pterins in which case fluorescent color becomes a basis for segregating but not identifying pterins.

Pterins are oxidized with loss of fluorescent properties by a permanganate in acetic acid media. Oxidation of pterins is used in the "murexide" test (so-called by analogy with murex, because the product is purple). This test was first applied to uric acid and its derivatives. Various characteristic intensities are given by leucopterin, xanthopterin, fluorescyanin, erythropterin and chrysopterin.

Pterins are sensitive to the sulfuric acid color test characteristic of carotenoids. They also give a blue test with the concentrated acid, thus careful separation of the two pigment classes must be ensured before the test can be diagnostic for carotenoids.

Pterins and ommochromes are the pigments found in the eyes of Drosophila. Extensive genetic research on these insects has resulted in refined thin layer chromatographic separations, the

prefered method for pterin separations. Mertens and Bennett (1969) and Hadorn (1962) outline several methods of thin layer chromatography with separated pterins "identified" by differences in fluorescence of the spots.

Color Production and Variation. The basis of this study is the suggestion of a malfunction in a biosynthetic pathway. This approach to the problem has become routine since G.W. Beadle and E.L. Tatum proposed the one-gene-one-enzyme hypothesis stating that genes control cell chemistry by controlling enzyme production, the biological chemical catalysts.

Pigment formation in Bettas should be similar to that of other organisms. A synthetic pathway for Drosophila eye pigment formation was reported by Ziegler (1961) and by Hadorn (1962). Ommochrome formation involves a progression of biosynthetic steps. The intermediates may be yellow, red, brown or black; mutants exist which block the sequence resulting in the varied color phenotypes.

Ommochromes occur only in invertebrates, but Drosophila eye pterins also exhibit alterations in synthetic pathways which result in mutant varieties. An example is "rosy." Wild type flies have isoxanthopterin, uric acid and 2-amino-4-hydroxypteridine. The rosy mutant lacks isoxanthopterin and uric acid. It has 2-amino-4-hydroxypteridine and, in addition, hypoxanthine.

This example shows how one gene disorder can affect diverse

compounds even though the mutation affects only one enzyme forming system. This same process is suspect in pterin formation in the red-yellow mutant forms of Bettas.

Although it is not easy to isolate and identify various animal pigments, it is much more difficult to discover how the animal produces them. Pigments are complex organic compounds and their synthesis from a few simple inorganic substances is difficult to follow. However, with the combined efforts of two methods, biochemical and genetic, the metabolic pathway for pigment formation can be deduced. A complete synthesis route would include the step-by-step linkages of the groups forming the complex molecule determined by radioisotope labeling. However, valuable information results from analysis of only the end products in mutant varieties where slight modifications of chemical components display phenotypic differences in pigmentation.

Lucas (1968), in his study of color mutants of Bettas, reported that

> "Red pigment in wild Bettas is limited to portions of the caudal, anal, and pelvic fins and a patch on the operculum. It is much more obvious in males. In domestic stocks, I found it to deviate in three distinct ways: a total absence, an extension to include the entire fish, and a variegated or "piebald" effect. These were observed in various combinations with other color elements. In some combinations red may be hidden."

Phenotypically, if combined with a lack of dark pigment (cambodia) the first variation produces a light non-red that

fanciers call "yellow," "pastel" or "white." The extension of red to include both body and fins produces a variety of phenotypes refered to as "blood-red," "tomato red," "bright red," "lavender" and "purple." The pastel and bright red are the mutants selected for this study and are pictured in Figure 3.

Lucas' (1968) genetic studies show the non-red abnormality to breed true. Outcrosses of non-red females and normal males show the non-red to be clearly recessive. Further data indicated no linkage between the cambodia and non-red loci. The extended red abnormality almost always breeds true. Since outcrosses of red x normal produce red progeny, a dominant mutant is implied.

Wallbrunn (1951) suggests that red pigment formation in Bettas involves at least two loci, one controlling body pigmentation, the other the fins, and that one gene has variable penetrance and expressivity.

Lucas (1968) also considered yellow color variations and found that "yellow" phenotypes depend upon the absence of black and red coupled with an increase in yellow. He concluded that "yellow" may be an alteration of red resulting from a blocked synthetic pathway that leaves an intermediary (yellow), or alternately, that more than one pigment is involved. If the latter is true of yellow mutants, then one pigment could be absent and the other changed to yellow. However, either possibility suggests a strong dependence upon the red genotype.

MATERIALS AND METHODS

In this study, designed to show the presence (or absence) of carotenoid and/or pterin pigments in red and yellow mutant stocks of Bettas, two analytical methods were employed:

(1) The methods of Sumner and Fox as outlined by Goodrich, et al. (1941) used different solvents to extract the various pigments. These are then identified by chromatographic techniques.

(2) Modification of methods for pterin analysis, as outlined by Fox and Vevers (1960) and D. Fox (1953), and carotenoid analysis as outlined by Goodwin (1955) were used. Various chemical and physical techniques were used, including spectral analysis, chromatographic separations, and identification by fluorescence.

Plant pigments were used as standard references for the carotenoids, Drosophila eye pigments for the pterins. Separations were made by thin layer and column chromatography.

Chromatographic methods provide a "gentle" means of separation, isolation and identification. Their applications are extensive in biological research. They are particularily useful for organic materials that are easily decomposed, and provide an analytical technique for separation on a micro or semi-micro scale, a factor often important in biology (Stein and Moore, 1951; Stock and Rice, 1967).

Thin layer and column absorption were used in this research. In both, a solid stationary phase functions as an adsorbent for

a moving liquid phase. In thin layer chromatography (TLC) a thin adsorbent layer is spread over a glass plate (commercially prepared sheets are available), while in column chromatography the adsorbent is a column held in a glass tube.

Adsorption chromatography is sensitive to steric or spatial differences between solutes; it is sensitive to slight differences in stereochemistry. It is also handy, especially in column form, for separations of solute mixtures varying in polarity and structure (Bobbitt, Schwarting and Gritter, 1968).

For both adsorption techniques silica gel and alumina (aluminum oxide) are the adsorbents most commonly used. The solvents, or elutants, vary depending on the polarity needed to dissolve and move a sample component. For this research, adsorbent and solvents were selected with reference to those used as standards.

The first chromatographic techniques were used to separate highly colored compounds (hence the prefix "chromo-":color). Today, however, with the aid of various developing techniques, this analytical method has been extended to include a vast number of colorless compounds. Bobbitt, et al. (1968) and Gelman (1968) outline two frequently used visualization techniques. Iodine vapor will produce brown spots on a yellow background for most organic compounds, especially those that are unsaturated. The process is reversible. For many organic substances ultraviolet radiation produces colored fluorescing spots on a dark

background.

Calculation of R_f values gives a quantitative means of comparison for TLC separated components. The R_f value is the ratio of the spot migration to the solvent front migration and should be constant for the same compound provided experimental conditions are constant. The formula may be written:

$$R_f = \frac{\text{spot migration distance}}{\text{solvent front migration distance}}$$

The chromatographic procedures used in this research are outlined below:

I. Thin Layer Chromatography (Hadorn, 1962; Mertens and Bennett, 1969)

A. Two-dimensional thin layer chromatograms as outlined by Hadorn (1962)

1. Medium: Gelman ITLC Type A unactivated silica gel impregnated sheets, 20cm x 20cm

2. Developing Chamber:
   a.) Gelman Chromatography Chamber (white plastic)
   b.) Glass "sandwich" chamber

3. Solvent System:
   a.) 1st dimension: 28% NH_4OH:1-propanol 1:1 2 hours
   b.) 2nd dimension: 1-butanol:acetic acid (gl):water 20:3:7 2½ hours

4. Samples:
   a.) Standard (pterins): Squash 2 heads Drosophila melanogaster Swedish-B wild type
   b.) Unknown Sample: Squash freshly cut caudal fin sample (3mm x 3mm) for each mutant tested

5. Detection Methods:
   a.) Ultraviolet light - Blak·Ray UVL·21 (Fisher Sci.)
   b.) Iodine vapor
   c.) 3-5 drops $KMnO_4$-acetic acid medium on fluorescing spots

B. One-dimensional thin layer chromatograms based on Hadorn (1962) and as outlined by Mertens and Bennett (1960)

1. Medium:
   a.) Gelman ITLC Type A unactivated silical gel impregnated sheets, 20cm x 20cm (3 samples per sheet
   b.) Whatman No. 1 Filter Paper (Chromatography Paper)

2. Developing Chamber:
   a.) Gelman Chromatography Chamber
   b.) 2 lb. coffee cans with plastic lids
   c.) Glass "sandwich" chamber

3. Solvent System:
   a.) Pterins
      1.) 28% NH_4OH:1-propanol 1:1 1½-2 hours
      2.) 1-butanol:acetic acid (gl):water 20:3:7 1½-2 hours
      3.) 28% NH_4OH:1-propanol 40:60 2 hours
   b.) Carotenoids
      1.) Chloroform:Ethanol 97:3 ½-1 hour

4. Standards:
   a.) Pterins: Squash 2 Drosophila heads
   b.) Carotenoids: Carotenoids TLC results from Mohrig and Neckers (1968)

5. Unknown Samples:
   a.) Squash freshly cut caudal fin sample (3mm x 3mm) for each run
   b.) Spot solution samples from petroleum ether, acetone, methanol extracts and boiled water and ammonia solutions from carotenoids separation solutions

6. Detection Methods:
   a.) Ultraviolet light
   b.) Iodine vapor
   c.) 3-5 drops $KMnO_4$-acetic acid medium on fluorescing spots

II. Column Chromatography (Goodwin, 1955)

A. Column Packing: Alumina, Merck; unactivated, 8 gm. Wet-pack using petroleum ether

B. Solvent System: (Goodwin, 1955)

1.) Petroleum ether
2.) Ether
3.) Acetone
4.) Benzene
5.) Chloroform
6.) 1,2-Dichloroethane
7.) Ethanol
8.) Methanol

(All elutants reagent grade)

C. Unknown Sample: Prepare sample based on method of Sumner and Fox (Goodrich, et al., 1941)

1.) Put fish in boiling water and let set for 5 minutes to help "set" the carotenoids
2.) Grind skin and scales with acetone
3.) Spot some acetone extract for TLC check for carotenoids
4.) Extract acetone solution with petroleum ether and saturated salt water
5.) Extract petroleum ether layer with methanol using equal quantity of methanol and petroleum ether; add water to dilute alcohol to 90% until methanol layer separates

Pigments in Petroleum Ether	Methanol
Carotenes	Xanthophylls
Xanthophyll esters	
Astacene	

6.) Spot petroleum ether layer for TLC check for carotenoids
7.) Spot methanol layer for TLC check for carotenoids
8.) Color test methanol layer with sulfuric acid (conc.) for xanthophylls
9.) Run petroleum ether layer through wet-packed adsorption column using petroleum ether as first elutant
10.) Elute column with solvents (above) in order
11.) Examine each eluted solvent visually, with ultraviolet light and spectrophotometrically
12.) Spectrophotometric examination
    a.) Evaporate eluting solvent and redissolve in spectrally pure chloroform
    b.) Using Beckman.DB spectrophotometer and recorder and Beckman silica cells (10mm, Class AA), examine samples in both ultraviolet and visible regions
        1.) Rate: 10 mμ /mm 60 cycles
        2.) Lamps: Tungsten (visible) 320-800 mμ
            Hydrogen (ultraviolet) 200-340 mμ
    c.) Compare absorption values to those listed by Goodwin (1955)
13.) Color test with conc. sulfuric acid the eluted fractions dissolved in chloroform
14.) Extract skin residue with 20% NH_4OH
15.) Spot and TLC to test for pterins
16.) Add $KMnO_4$-acetic acid media to original boiled water

D. Detection Methods:
   1.) Collect fractions watching for colored bands and color in the eluted solvent
   2.) Ultraviolet light
   3.) Spectrophotometric analysis

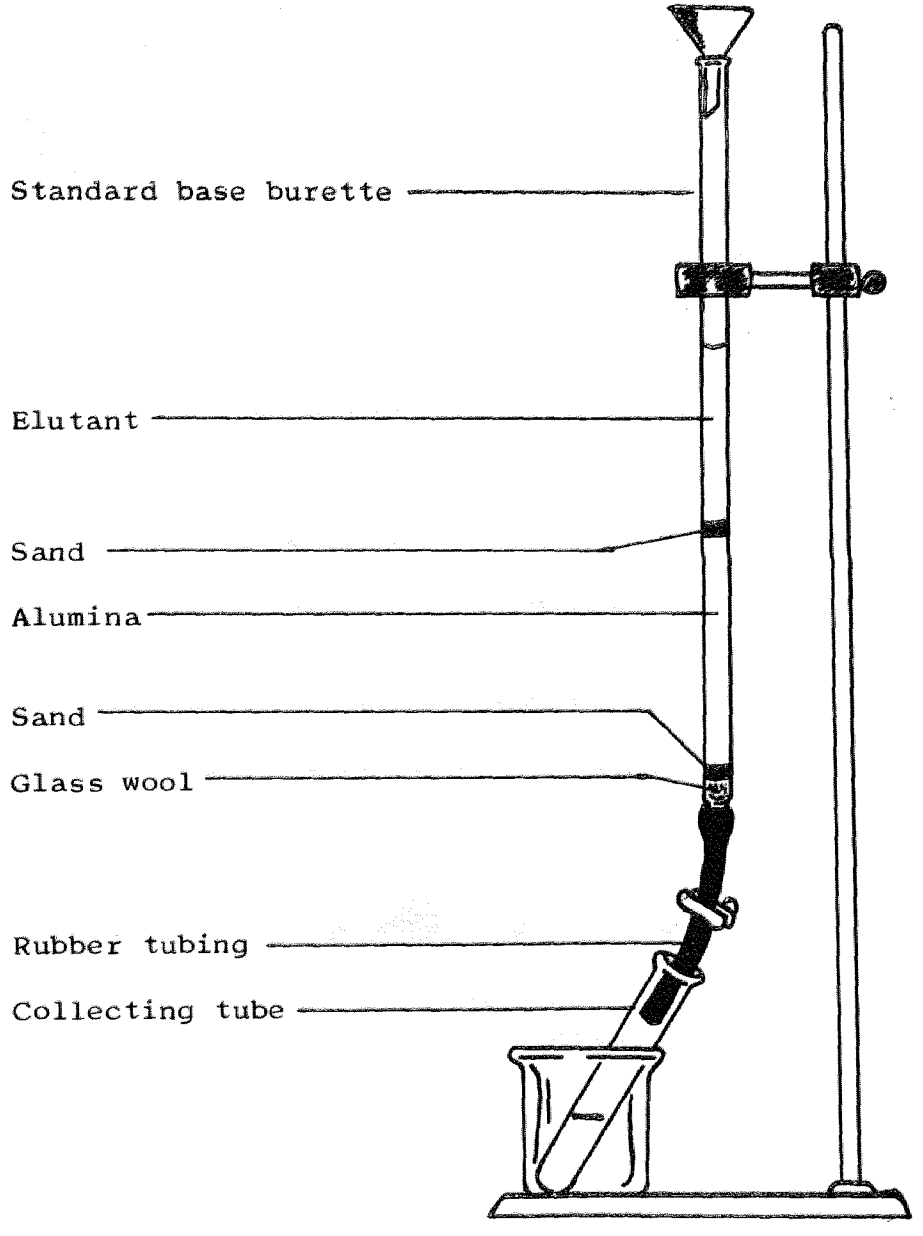


Figure 11. Apparatus for column chromatographic separation of carotenoids.

Pterins in skin tissue were tested for fluorescence after protein bonds were broken using dilute base. This proceded as follows:

1. Using scissors, remove the dorsal fin and observe microscopically.
2. Test for fluorescence using Blak·Ray UVL·21 ultraviolet light.
3. Add 3-5 drops 20% NH_4OH; observe with ultraviolet light.
4. Add 5-10 drops $KMnO_4$-acetic acid media (two grains dissolved in 10 ml 6-N acetic acid); observe with ultraviolet light.

Sodium fusion with an elemental test for nitrogen was also tried on fin material cut from the fish. The specific method employed was that outlined in Experiments in Organic Chemistry (Mohrig and Neckers, 1968).

The "murexide" test outlined in Practical Physiological Chemistry (Hawks, Oster and Summerson, 1947) was tried on freshly cut fin material. Concentrated nitric acid (2-3 ml) was poured on the fin and gently heated evaporating to dryness. A yellow residue remains upon cooling. Very dilute ammonium hydroxide was added which should cause the residue to turn purplish-red indicating a positive test as murexide (=ammonium purpurate) is formed. Uric acid was used as a standard.

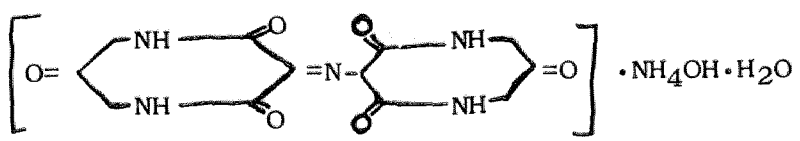


Figure 12. Murexide.

Suspected carotenoid solutions were also tested in several ways. Oxidation resulting in color loss should occur if they are left exposed to air and sunlight. A green-blue color upon addition of concentrated sulfuric acid is a positive test for carotenoids dissolved in anhydrous, ethanol-free, chloroform. Fox and Vevers (1960) also note that goldfish skin extracted repeatedly with acetone produced a golden-yellow solution as the carotenoids were dissolved; this same procedure was used on the Bettas.

RESULTS AND DISCUSSION

This experiment was an attempt to show that the yellow Betta phenotype is a mutant resulting from improper or inadequate red pterin synthesis. It is believed that red and yellow color in Bettas is caused by pterins instead of or along with carotenoids as reported by Goodrich, et al. (1941) who reported results for a red mutant in which erythropterin was detected and a yellow with only lutein.

The assumption is made that the red mutant merely represents a quantitative increase in pigment in the pigment cells; a qualitative difference is predicted for the yellows with either different pterins or a pterin-carotenoid mixture being present.

The presence of lutein only would suggest suppression of pterin synthesis unmasking the lighter carotenoids and/or pterins.

If pterins are detected, then the hypothesis of a qualitative change in the yellows is supported indicating either an incomplete or suppressed pterin synthesis or production of new pterin(s) by a new synthetic route.

The 1-dimensional TLC results of the pterin analysis are presented in Figures 13 and 14. R_f values and averages for the Drosophila standard and the red and yellow Betta mutants are stated in Tables 1 and 2.

Comparison of the two solvent systems used shows poor separation of the Drosophila (standard) pterin eye pigments. The propanol-ammonia solvent gave better separation showing three color regions distinctly when ultraviolet light was used. The red spot (R_f=.07) was clearly visible without ultraviolet light and fluoresced red with it; this spot turned yellow-brown with iodine vapor developer. This spot corresponds to Hadorn's (1962) visible and fluorescing (red-)orange spot characteristic of drosopterins.

The bright blue fluorescing spot (R_f=.09) corresponded to isoxanthopterin; possibly this spot represents a mixture of the violet-blue fluorescing isoxanthopterin and green-blue fluorescing xanthopterin since the later does not appear independently.

The green fluorescing streak (R_f=.19-.75) probably represents a mixture of yellow fluorescing sepiapterin, blue fluorescing 2-amino-4-hydroxypteridine, biopterin and yellow fluorescing isosepiapterin, where combined blue and yellow fluorescence

Drosophila

SAMPLE	RED	RED-BLUE	GREEN	GREEN STREAK
D-1	.07	.09	.18	.20-.77
D-2	.07	.09	-	.18-.71
D-3	.06	.09	.17	.18-.78
D-4	.07	.09	-	.19-.75
D-5	.08	.10	-	.20-.76
Average	.07	.09	.18	.19-.76

Red Bettas

SAMPLE	RED	GREEN	BLUE	GREEN	BLUE
R-1	.16	.20	.36	.57	.75
R-2	.06	.20	.40	.53	.75
R-3	.04	.20	.37	.56	.69
R-4	.06	.18	.40	.46	.74
R-5	.04	.20	.31	.55	.74
Average	.05	.20	.37	.54	.74

Yellow Bettas

SAMPLE	GREEN	GREEN STREAK	RED SPOTS
Y-1	.14	.19-.64	(3) .30
Y-2	.09	.13-.62	-
Y-3	.13	.00-.64	(1) .21
Y-4	.14	.15-.56	-
Y-5	.13	.06-.52	-
Average	.13	.16-.59	-

Table 1. R_f values of fluorescent pterins from 1-dimensional chromatographic separation for 1:1 1-propanol:28% NH_4OH.

Drosophila

SAMPLE	RED STREAK	BLUE STREAK
D-1	.11	.35
D-2	.14	.54
D-3	.11	.45
D-4	.10	.38
D-5	.09	.30
Average	.11	.40

Red Bettas

SAMPLE	RED	BLUE STREAK	GREEN	BLUE
R-1	.08,.20	.00-.83	-	-
R-2	.12	.23-.60	-	.70
R-3	.15	.25-.68	.75	.85
R-4	.11	.35 (green)	.50	.67
R-5	.11	.30 (green)	.48	.64
Average	.11	.24-.64;.33	.57	.72

Yellow Bettas

SAMPLE	RED	BLUE STREAK	BLUE
Y-1	.08	.38 (spot)	.60
Y-2	.12	.39 (spot)	.70
Y-3	.15	.14-.65	-
Y-4	.11	-	.50
Y-5	.11	.00-.62	-
Average	.11	-	.60

Table 2. R_f values of fluorescent pterins from 1-dimensional chromatographic separation for 20:3:7 1-butanol: acetic acid:water.

produced the observed green fluorescent color.

The reason(s) for poor separation are unknown although one factor may be in the wild-type Drosophila standard used in this experiment. A strain known as Swedish-B was available which may differ from Hadorn's. Chromatographic information was not available for the Swedish-B strain. For this reason, the results presented by Hadorn (1962) were used as standard and are given in Figure 15.

The red pigments were rather well separated using the propanol-ammonia solvent. (Figure 13.) The red fluorescing spot (R_f=.05)was also clearly visible without ultraviolet light and corresponds to drosopterins in the standard. This spot turned yellow-brown with iodine vapor developer. All other spots were visible only with ultraviolet light. The green (R_f=.20) faded when the paper was completely dry. Possibly the material in this spot was easily oxidized with loss of fluorescence, or it moved up the paper combining with those of the blue spot (R_f=.37).

The blue spot corresponded to the violet-blue fluorescing isoxanthopterin. The green spot (R_f=.54) corresponded to the green-blue fluorescing xanthopterin, while the final blue spot (R_f=.74) could be either 2-amino-4-hydroxypteridine or biopterin or a mixture of these. The yellow fluorescing sepiapterin and isosepiapterin correspondents were absent in the red mutant chromatograms.

Iodine developer caused the entire chromatogram to show

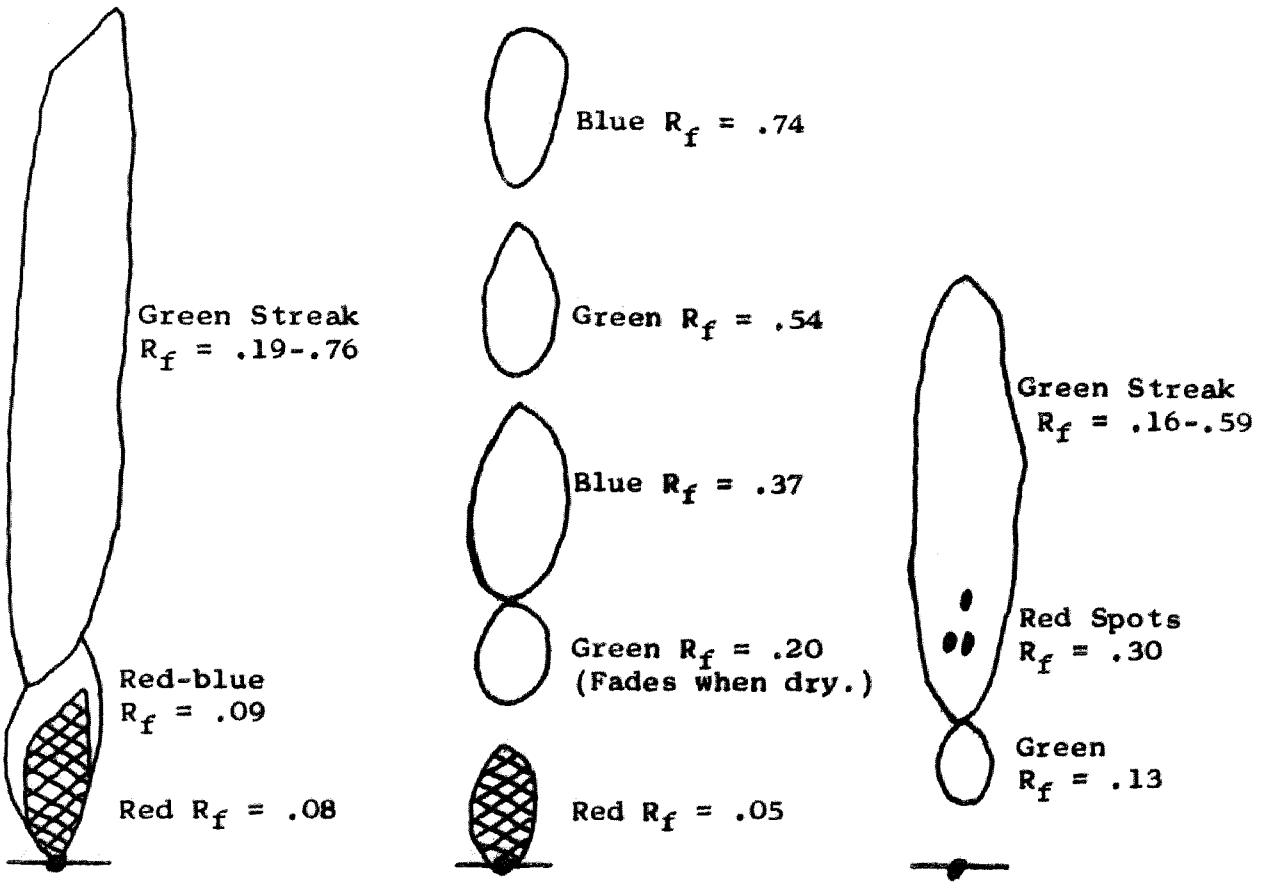


Figure 13. 1-Dimensional pterin chromatograms for 1:1 1-propanol:28% NH_4OH.

yellow-brown with darker spots occurring at the red and green spots (R_f=.05,.20). No new spots were observed with iodine vapor developer.

The yellow mutant also showed distinctive separation. (Figure 13.) All spots were fluorescent and none showed without ultraviolet light. Appearing first was a green spot (R_f=.13), then a green streak (R_f=.16-.59). The first green spot does not correspond to anything thus far mentioned. Its R_f value was closest to the drosopterins, but its fluorescent color was more like the xantho- or isoxanthopterins. Iodine vapor developer caused the entire chromatogram to become brown-yellow with a dark spot at R_f=.13. In only one of the fish tested (Y-5) did this R_f=.13 spot appear blue rather than green. This spot also showed increased amount of fluorescence when compared to the red mutant's fluorescence.

Unique in the yellow mutant were three red fluorescing spots occurring at R_f=.30 for fish Y-1 and one red fluorescing spot at R_f=.21 for fish Y-3. Fish Y-1 also showed blue fluorescence in the sample spot. These red fluorescing spots have R_f values much higher than the drosopterins and their fluorescent color does not compare to other pterins.

As with the propanol-ammonia solvent system, separation of the Drosophila (standard) pterin eye pigments was also poor with the butanol-acetic acid-water system. Two streaks were produced as shown in Figure 14. The red streak (R_f=.11) was colored with

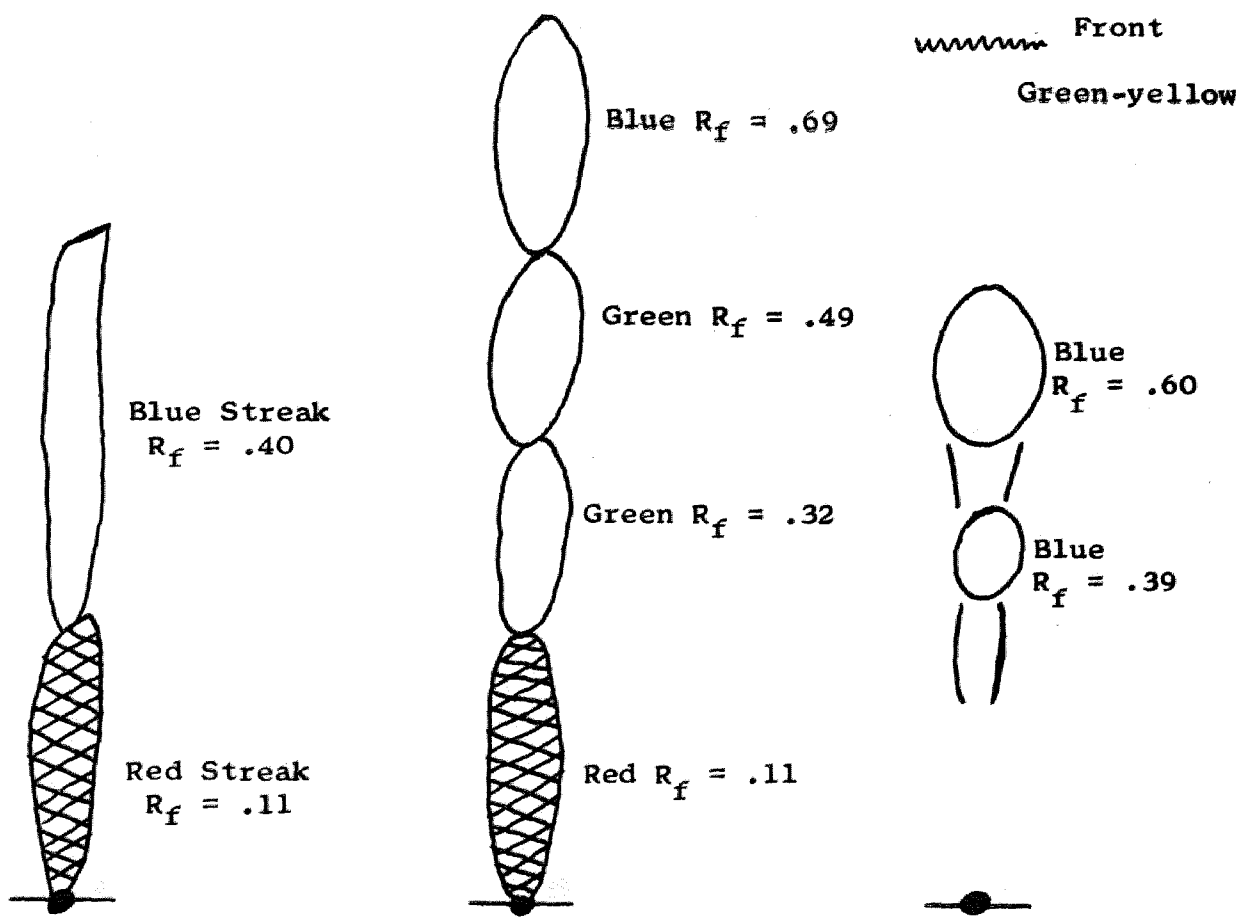


Figure 14. 1-Dimensional pterin chromatograms for 20:3:7 1-butanol:acetic acid:water.

and without ultraviolet light and would represent the drosopterins possibly combined with the xanthopterins. The blue fluorescing streak (R_f=.40) also represented a mixture of pterins.

The red mutant gave a chromatogram similar to that obtained with the propanol-ammonia system, with modification in R_f values as would be expected with a change in solvent system. The visible and fluorescing red spot occurred more as a streak (R_f=.11), while a definite green fluorescing spot occurred at R_f=.32. (In the other solvent system this appeared at R_f=.20 and faded when the paper dried.) A blue fluorescing streak (R_f=.21-.68) separated this green spot and the next green fluorescing spot at R_f=.49. These were comparable to the blue (R_f=.37) and green (R_f=.54) indicated in the propanol-ammonia system. Finally, the blue fluorescing (R_f=.69) spot was similar to the blue (R_f=.74) previously noted.

The yellow mutant showed a modified chromatogram. Previously, spots and streaks were green fluorescent; the fluorescent color is now blue. A blue streak appeared with definite spots at R_f=.39 and R_f=.60. A green-yellow fluorescence also sppeared in the solvent front indicating the solvent was too polar and components of the mixture were not able to separate properly.

Iodine vapor developer produced brown-yellow staining in the red drosopterins region on the Drosophila and red mutant chromatograms and in the solvent front for the yellow mutant.

Complete pieces of fin tissue were used for 1-dimensional

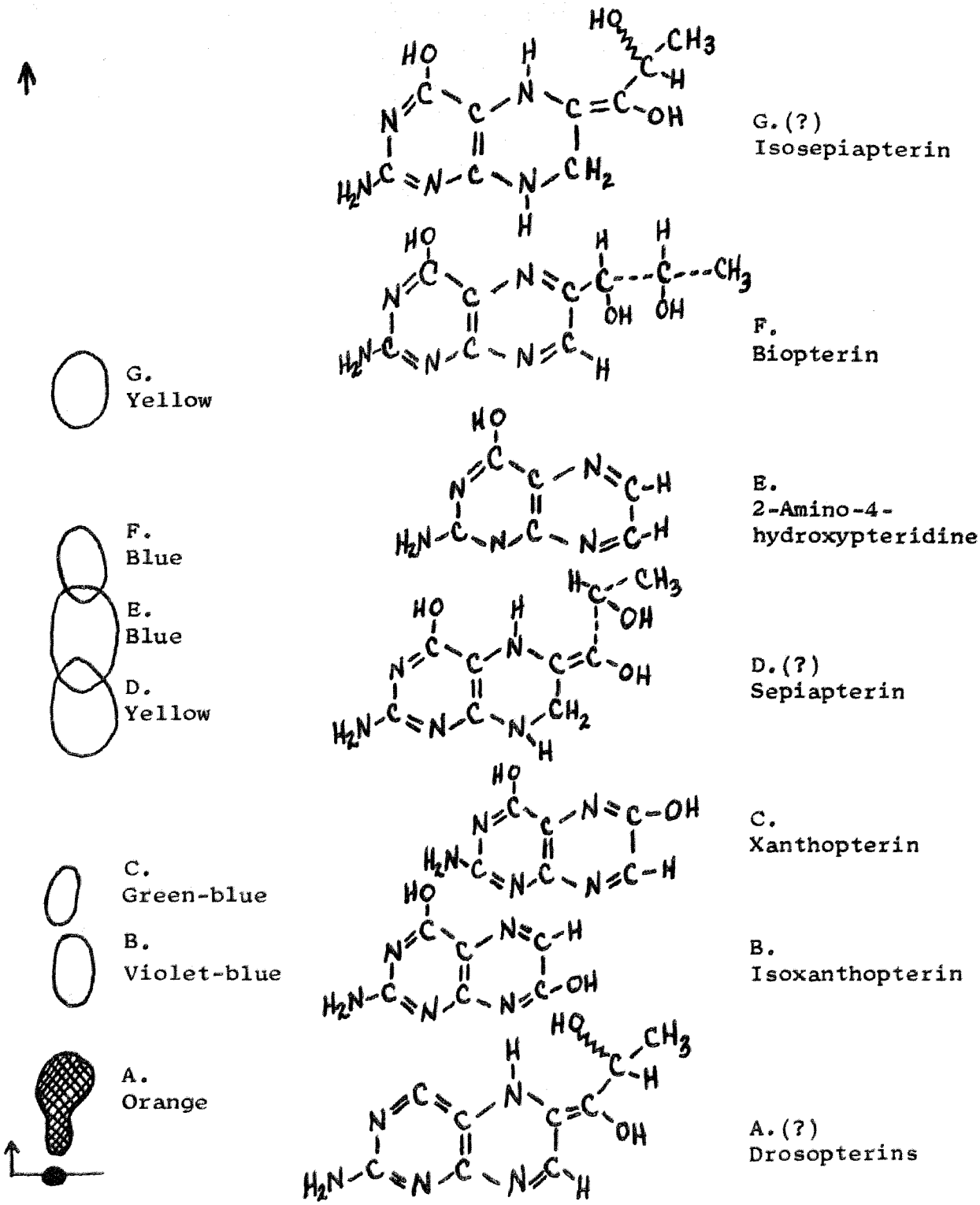


Figure 15. Drosophila eye pterin chromatogram and structures from Hadorn (1962).

thin layer chromatograms indicating the possibility of both pterin and carotenoid pigments being present. However, pterin separation from carotenoids was based on the solvent system where pterins are soluble in the polar system and carotenoids, being soluble only in organic solvents, were left in the tissues.

Two dimensional TLC results are presented in Figures 16, 17 and 18, with R_f values and averages listed in Table 3. As might be expected from the 1-dimensional TLC results, the two-dimensional Drosophila (standard) chromatograms did not give results reported by Hadorn (1962). Four of the six fluorescing spots, or regions, could be detected, but their chromatographic positions were greatly altered.

The R_f values reported are relative to the second solvent front. Accordingly, for the Drosophila, a visible and fluorescing red extended toward the first solvent front with R_f=.00; a faint blue to blue-green fluorescing streak was beside the red, it also having R_f=.00. The red represents drosopterins, while the blue probably represents 2-amino-4-hydroxypteridine and/or biopterin, possibly combined with xanthopterin. A blue fluorescing spot (R_f=.02) appeared above the sample spot corresponding to isoxanthopterin.

A bright yellow fluorescing irregular shaped streak appeared almost at the intersection of the two solvent fronts. With R_f=.96, this region corresponds to sepiapterin and/or isosepiapterin. A yellow-green fluorescence was observed in the second solvent front

Drosophila

SAMPLE	RED	BLUE (faint)	BLUE	YELLOW
D-1	.00	.00	.02	.98
D-2	.00	.00	.02	.97
D-3	.00	.00	.03	.98
D-4	.00	.00	.02	.88
D-5	.00	.00	-	.98
Average	.00	.00	.02	.96

Red Bettas

SAMPLE	BLUE	RED	BLUE	GREEN	BLUE	YELLOW
R-1	.00	.15	.55	.74	.89	.98
R-2	.00	.15	.55	.74	.74	.99
R-3	.00	.12	.43	.60	.65	.89
R-4	.00	.15	.59	.60	.66	.95
R-5	.00	.20	.76	.70	.60	.95
Average	.00	.15	.58	.66	.71	.97

Yellow Bettas

SAMPLE	(BLUE)*	(RED)*	BLUE	BLUE	GREEN
Y-1	-	-	.17	-	.98
Y-2	.00	.01	.25	.70	.98
Y-3	.00	.01	.20	.71	.99
Y-4	.00	.01	.25	.71	.99
Y-5	.00	.00	.25	.71	.99
Average	.00	.01	.22	.71	.99

*Fades when dry

Table 3. R_f values of fluorescent pterins from 2-dimensional chromatographic separation.

indicating a solvent polarity too high to separate and then release all components of the mixture.

Iodine developer gave a brown spot at the red (R_f=.00) region and causes dark yellow-brown coloration of the yellow (R_f=.98) streak and the second solvent front.

Deviations from the expected results were again probably caused by a difference in the strain of wild-type fly used (Swedish-B).

The red mutant gave rather distinctive separations. (Figure 17.) A red streak visible with and without ultraviolet light had R_f=.15 corresponding to drosopterins. All other spots were visible only with ultraviolet light.

A blue spot occurred at R_f=.00 but did not coincide with the blue (R_f=.00) of the standard since it was not displaced to the right but instead was next to the sample spot. Possibly this corresponds to the fading green fluorescent spot (R_f=.20) observed in the 1-dimensional TLC results. These results further support the idea that the materials of this spot do not correspond to anything reported in the standard therefore suggesting the presence of a new substance.

Another blue spot occurred at R_f=.58 corresponding to isoxanthopterin, the faint green spot (R_f=.66) represented xanthopterin, and the blue spot (R_f=.71) corresponds to 2-amino-4-hydroxypteridine and/or biopterin. A bright green streak occurred at the intersection of the two solvent fronts. With R_f=.97, it

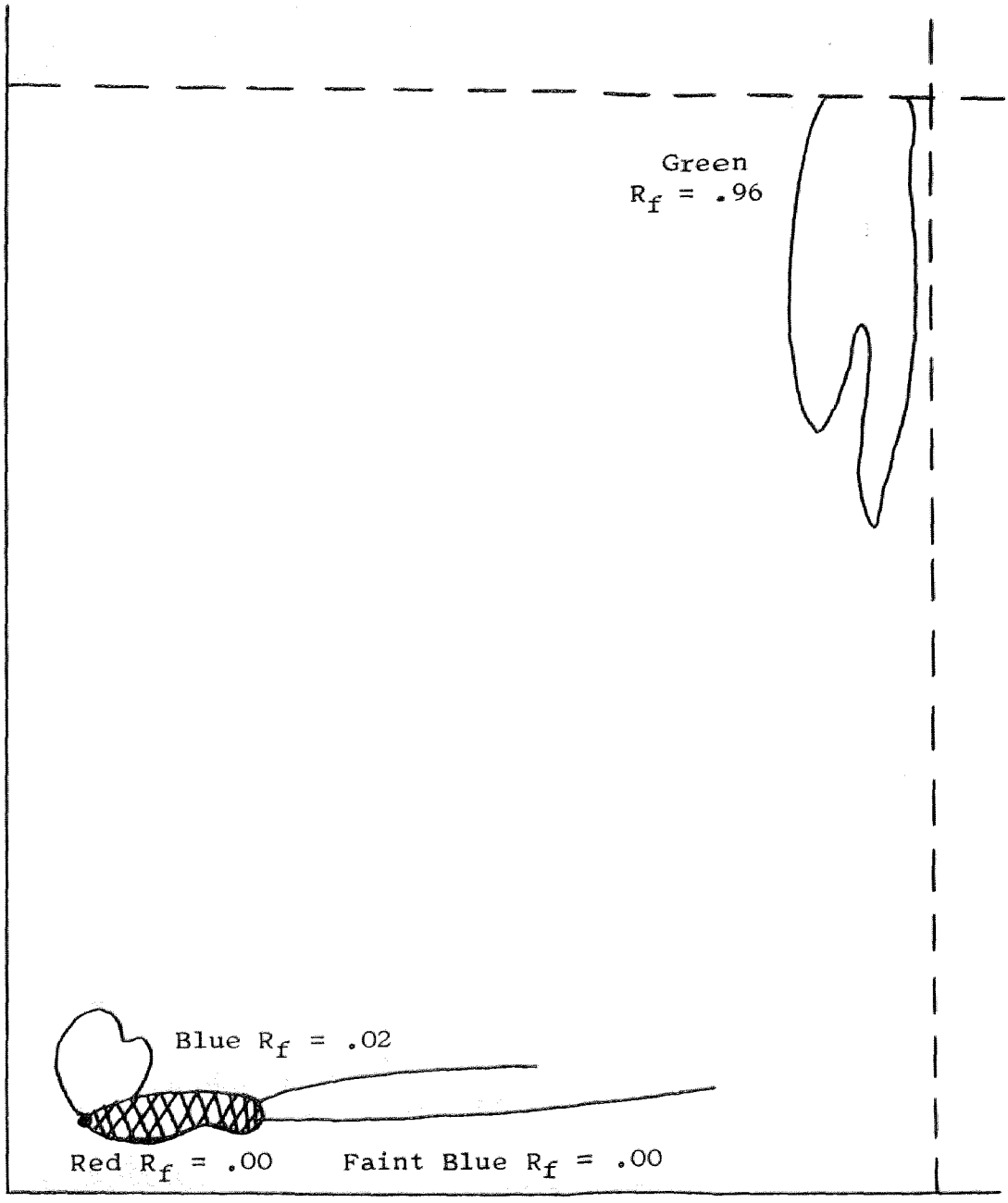


Figure 16. 2-Dimensional pterin chromatogram of Drosophila melanogaster.

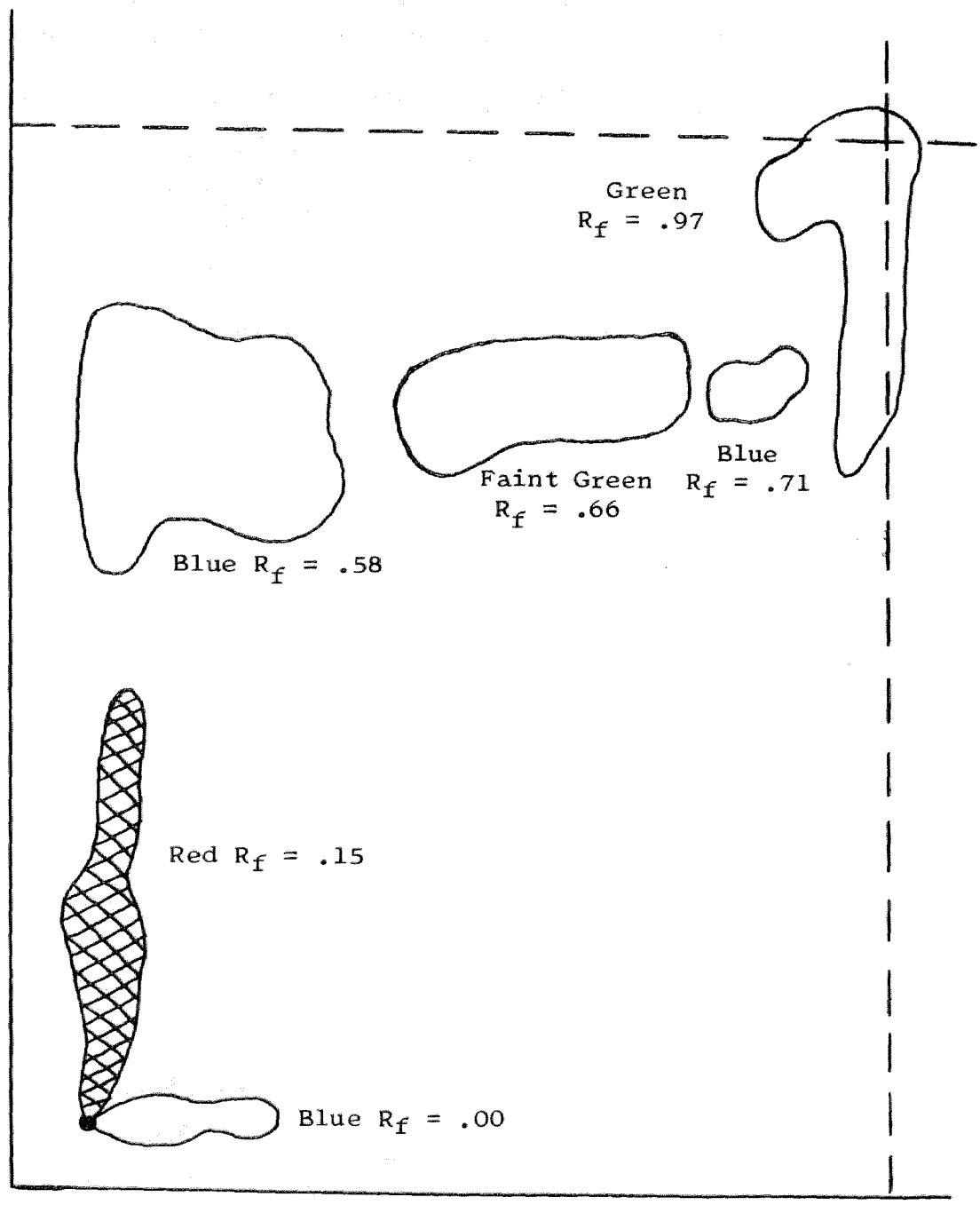


Figure 17. 2-Dimensional pterin chromatogram of red mutant, Betta splendens.

corresponds to sepiapterin and/or isosepiapterin in the standard. It should be noted that a corresponding yellow-green spot did not occur in the 1-dimensional TLC, probably because this material was carried with the solvent to the front.

Iodine vapor developer caused dark yellow-brown coloration of the red and green spots (R_f=.15,.95).

The 2-dimensional TLC results for the yellow mutants were also distinctive but not quite as consistant as those of the red. (Figure 18.) All spots were visible only with ultraviolet light.

A small red fluorescing spot (R_f=.01) and a blue fluorescing spot (R_f=.00) appeared when the chromatogram was still wet but faded as it dried. A blue streak with R_f=.22 occurred in the same position as the red streak in the red mutant.

A faint blue streak (R_f=.71) occurred in the same positions as the faint green (R_f=.68) and blue (R_f=.71) corresponding to isoxantho- and xanthopterin of the red mutant. A bright green to yellow-green streak appeared at the intersection of the two fronts (R_f=.99). Iodine developer caused dark yellow-brown coloration of this spot, similar to sepia- and/or isosepiapterin in the standard.

Individual chromatographic variations were the appearance of more blue fluorescing spots both above and below the R_f=.71 streak. These extra spots occurred at R_f=.37,.45 and .83, and usually faded when the chromatogram dried.

Figure 19 gives a composite drawing of the results for the

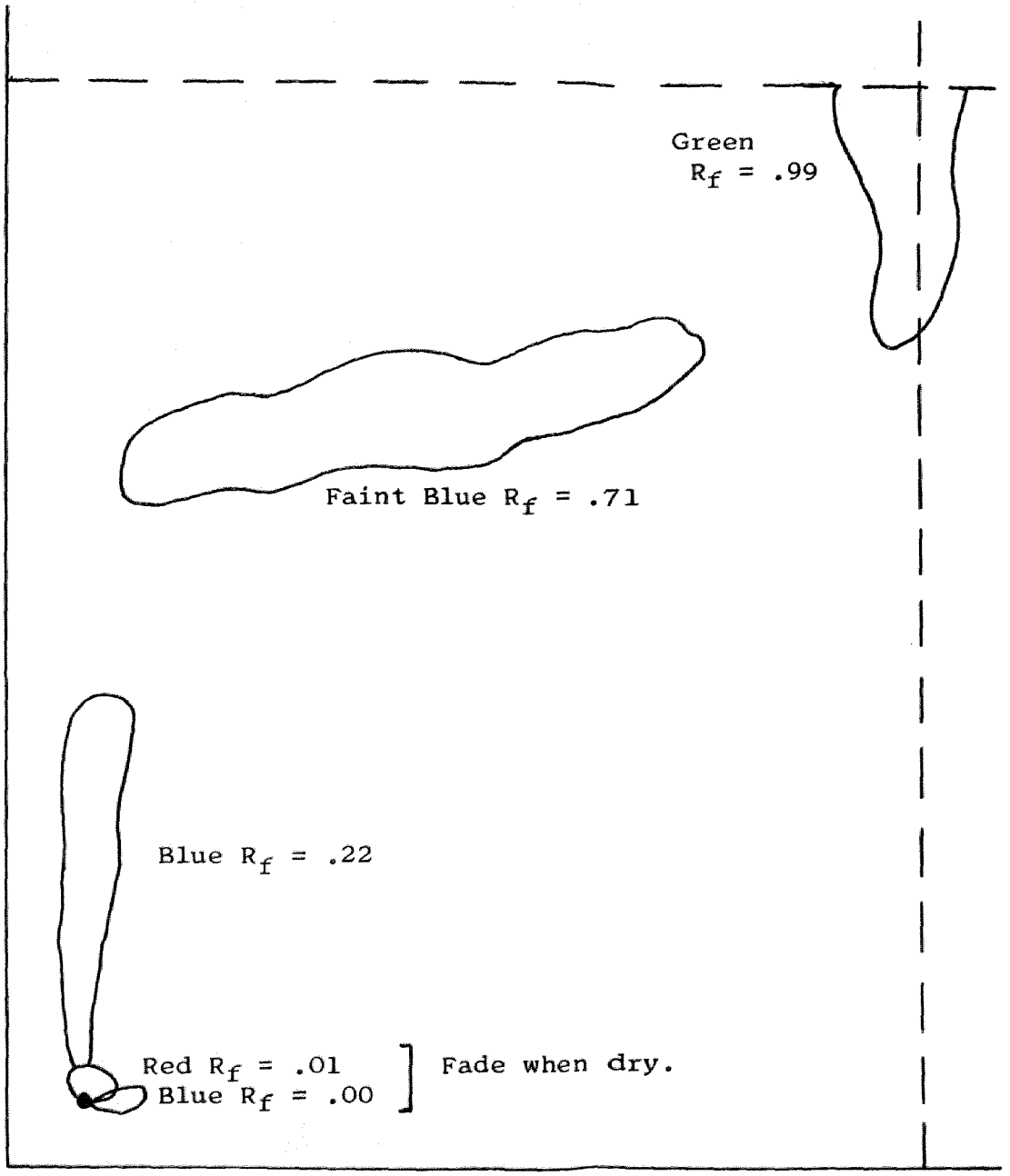


Figure 18. 2-Dimensional pterin chromatogram of yellow mutant, Betta splendens.

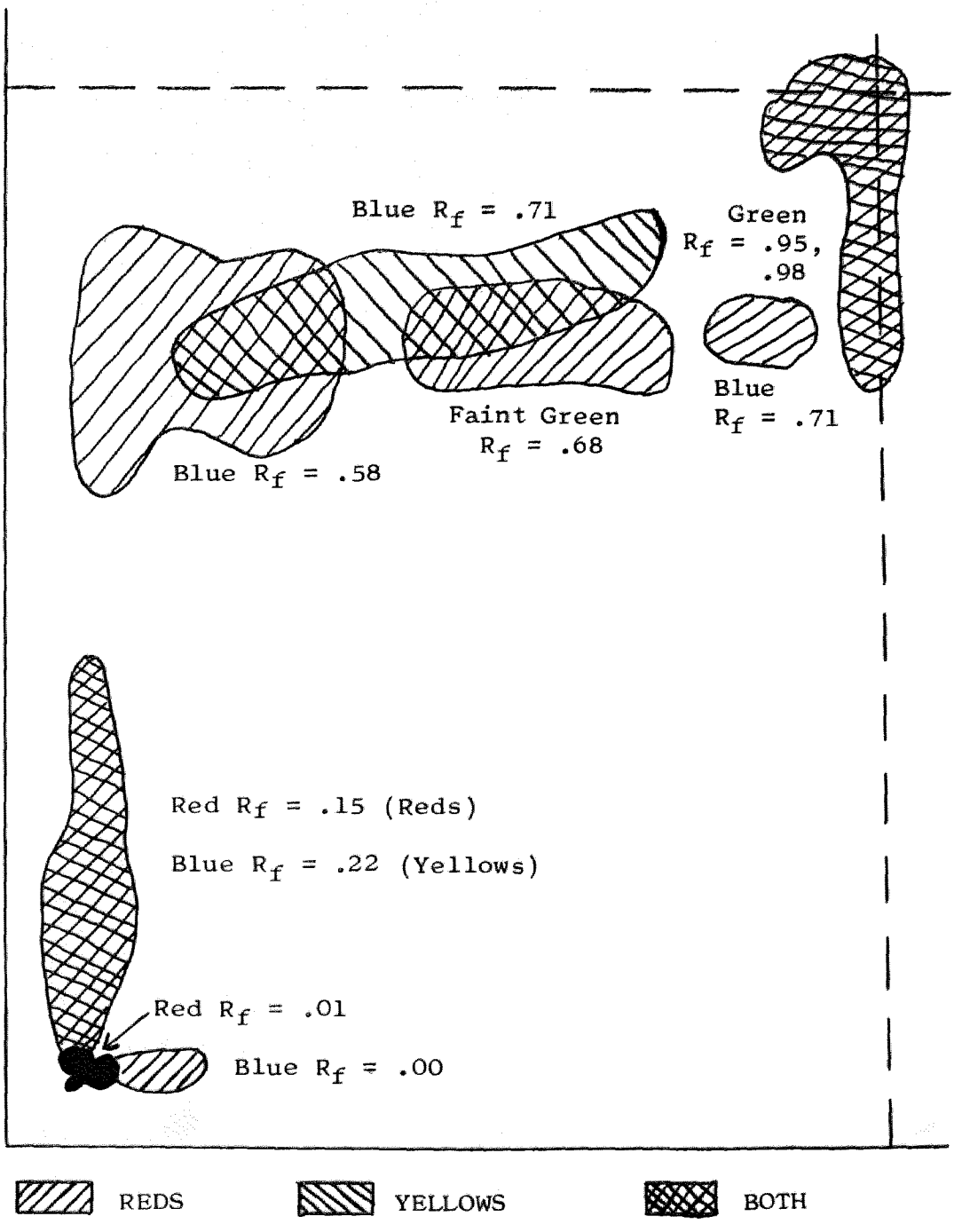


Figure 19. Combined 2-dimensional pterin chromatograms of red and yellow mutants, Betta splendens.

red and yellow mutants. This diagram strongly suggests an alteration in the red fluorescing drosopterins of the red mutant to a blue fluorescing substance, possibly an altered or intermediary form of isoxantho- and/or xanthopterin.

Both have isoxanthopterin and xanthopterin appearing at the standard chromatographic positions. The blue fluorescing 2-amino-4-hydroxypteridine and/or biopterin are both probably absent in the yellow mutant, while both contain the green to yellow-green fluorescing sepia- and/or isosepiapterin at R_f=.95 and .99.

Special test results also aided in confirming the presence of pterins. Dorsal fin microscopic observation of the red mutants showed red granular specks definitely lacking in black except for a minute number of black granules in one observed sample. Upon contact with air, oxidation occurred resulting in blackening of the red granules. The tissue showed no fluorescence with ultraviolet light. When 28% NH_4OH was added a blue fluorescing substance diffused into solution, while the boney fin rays fluoresced golden-red. With regular light the fluorescing material appeared yellowish-red and gelatinous; it surrounded the fin but the red granules were still present intact in the fin. Squashing the fin seemed to make no difference in the results, ie., the granules were still present surrounded by the fluorescing gel material.

The addition of permanganate-acetic acid media caused the

fluorescence to fade although not immediately (not characteristic of flavins). The red granules were left unchanged. The gelatinous material either turned yellowish-white and flocculent or became clearer and more colorless.

Dorsal fin microscopic observation of the yellow mutants showed no red granules. The tissues were very blue-green iridescent (guanine?) with very little actual yellow color. One sample showed a few red granules of pigment at the tip of the fin which became redder upon exposure to air. The tissue showed no fluorescence with ultraviolet light. When 28% NH_4OH was added two of the five fish tested showed no fluorescence and three of the five only a faint blue fluorescence. The tissue with the red pigment granules had faint green fluorescing material diffuse into solution. With regular light there was no noticeable change in the tissues and little or no gelatinous flocculent material was observed.

The addition of permanganate-acetic acid media caused fluorescence to fade when present. A slight amount of clear gelatinous material appeared above the fin. Squashing the tissues did not change the results.

The murexide test gave negative results as compared to the uric acid standard. The uric acid turned light yellow with addition of concentrated nitric acid and evaporation left a red-yellow residue. Addition of very dilute ammonia gave a red to blue-red color. The red mutant gave a yellow solution and residue

when concentrated nitric acid was added and then evaporated. All the fin tissue dissolved. Addition of very dilute ammonia resulted in a yellow color indicating a negative test. The same negative results were observed for the yellow mutant.

These results of the 2-dimensional chromatograms and special tests indicate:

1. Apparent modification of drosopterins from visible and fluorescing red form in the red mutant to a blue fluorescing substance in the yellow.

2. Isoxantho- and xanthopterin were present in both mutants.

3. Biopterin and/or 2-amino-4-hydroxypteridine were absent in the yellow mutant and present in the red.

4. Sepia- and/or isosepiapterin were present in both.

5. Possibly there was an easily oxidized substance that subsequently loses fluorescence present in the yellows.

1-Dimensional chromatograms and special test results indicate:

1. Complete removal of visible and fluorescing red drosopterins, or alteration to a green fluorescing substance with higher R_f value in the yellow mutant.

2. Probably xanthopterin and possibly isoxanthopterin were present in both mutants with a noteable increase in material in this region for the yellow mutant.

3. Biopterin and/or 2-amino-4-hydroxypteridine were absent in the yellow and present in the red.

4. Sepia- and/or isosepiapterin were absent in both.

5. Small red fluorescing spots with average R_f=.30 were present in some yellow mutants. This is much higher than the normal R_f=.05 or .08 for drosopterins.

Special pterin test results alone indicate:

1. No fluorescence until dilute ammonia was added then the red tissues fluoresced blue and the yellows fluoresced light blue or green.

2. Fluorescence faded with permanganate-acetic acid media.

3. Murexide tests were negative for both mutants.

4. Elemental tests for nitrogen were negative for both mutants.

Carotenoid determinations were to be based on the methods of Sumner and Fox as outlined by Goodrich, et al. (1941). Different solvents were used to extract and separate the two carotenoid classes after their initial separation from the pterins. The carotenes were dissolved into petroleum ether, while the xanthophylls were extracted into 90% methanol (10% water).

Chromatographic procedures were modified from those outlined by Goodrich, et al. (1941). Absorption maxima wavelengths reported by Goodwin (1955) for plant carotenoids were used as standards.

Table 4 lists the column chromatography results for both mutants. Most results were similar, but noteable differences did occur.

The petroleum ether eluted an orange band from the red mutants while a yellow band was eluted from the yellows. The methanol eluted a more viscous clear, colorless, green fluorecing substance from both mutants. Differences in viscosities in the 1,2-dichloroethane and methanol solvents occurred as consecutive elutants ran together in the same test tube.

Table 5 describes the residues left in the test tubes after the original elutants evaporate. When these residues were dissolved in spectroanalyzed chloroform, all yellow mutant residues appeared clear and colorless except the first (petroleum ether eluted) which was light yellow. The material which had been in the petroleum ether fraction of the red mutant became orange in chloroform, the second solution light golden orange, the third almost colorless and the remaining solutions colorless.

Using a Beckman·DB recording spectrophotometer, absorption curves were run for each residue in chloroform. This data is summarized in Table 6. The instrument was first standardized using only chloroform. This gave a sharp peak at 242 mμ.

This data showed constant absorption maxima at 342, 278 and 242 mμ for almost all solutions. Chloroform and methanol solutions of the red mutant showed almost standard spectra, each having maxima only at 342 and the 242 mμ standard. Scale expansion (4x) used on six of eight yellow mutant eluted solutions showed a maximum at 238 mμ ; non-expanded spectra did not show this peak.

ELUTANT	RED MUTANTS	YELLOW MUTANTS
Petroleum Ether	Initial sol'n. golden yellow. Orange band eluted giving golden yellow sol'n. No fluorescence.	Initial sol'n. yellow. Yellow band eluted giving yellow sol'n. No fluorescence.
Ether	Golden yellow eluted sol'n.; no fluorescence.	Faint yellow eluted sol'n.; no fluorescence.
Acetone	Clear, colorless non-fluorescing solution.	Clear, colorless faint blue fluorescing sol'n.
Benzene	Clear, colorless non-fluorescing solution.	Clear, colorless non-fluorescing solution.
Chloroform	Clear, colorless non-fluorescing solution.	Clear, colorless non-fluorescing solution.
1,2-Dichloro-ethane	Clear, colorless non-fluorescing solution.	Clear, colorless non-fluorescing solution.
Ethanol	Clear, colorless with green fluorescing more viscous clear band.	Clear, colorless with green fluorescing more viscous clear band.
Methanol	Clear, colorless non-fluorescing with sol'n. interface apparent.	Clear, colorless non-fluorescing with sol'n. interface apparent.

Table 4. Carotenoid column chromatography observations.

ELUTANT	RED MUTANTS	YELLOW MUTANTS
Petroleum Ether	Dark red-orange oil with few crystals present.	Orange-yellow oil with few crystals present.
Ether	Dark red-orange oil with very few crystals present.	Orange-yellow oil with few crystals present.
Acetone	Small amount of orange-yellow solid to oily substance.	Yellow to dirty white solid residue.
Benzene	Very small amount solid residue.	Yellow to dirty white solid residue.
Chloroform	Sparse white coating on test tube that forms "snowflake" crystal pattern.	Small amount yellow to dirty white oily residue with few crystals.
1,2-Dichloroethane	Faint white coating on test tube with few white crystals.	Small amount yellow to dirty white oily residue with few crystals.
Ethanol	Very few impure white crystals.	Small amount of yellow to dirty white residue on test tube.
Methanol	Very few impure white crystals.	Yellow to dirty white crystalline residue.

Table 5. Carotenoid column chromatography residues.

CAROTENOID	ABSORPTION MAXIMA: mμ
Lutein	428 456 487
Isolutein	428 456 487
Phytoene	265-295
Phytofluene	(No wavelengths given)
Astaxanthin	472 (petroleum ether; from Fox and Vevers, 1960)

Table 6. Standard carotenoid absorption maxima (chloroform) from Goodwin (1955).

ELUTANT	ABSORPTION MAXIMA: mμ							
STANDARD - chloroform				242				
	RED MUTANT				YELLOW MUTANT			
Petroleum Ether	470	342	278	242	342	278	242	(238)*
Ether	470	342	278	242	342	278	242	(238)*
Acetone		342	278	242	342	278	242	(238)*
Benzene		342	278	242	342	278	242	(238)*
Chloroform		342		242		278	242	(238)*
1,2-Dichloro-ethane		342	278	242		278	242	(238)*
Ethanol		342	278	242	342	278	242	
Methanol		342		242	342	278	242	

(*Scale expanded 4x)

Table 7. Absorption maxima results for red and yellow mutants, Betta splendens.

None of these values corresponded to those reported by Goodwin (1955), including those for lutein, the carotenoid reported by Goodrich, et al. (1941) to be present in yellow Bettas. Tables 6 and 7 list the standard values along with the experimental data.

Most outstanding of these results is the maximum at 470 mμ for the first two solutions of the red mutant, with this being considerably greater in the first than the second. Astaxanthin, reported by Fox and Vevers (1960), gives an absorption maximum of 472 mμ in petroleum ether which, when coupled with the orange color of the solution, strongly suggests the presence of this carotenoid.

The consistent 278 mμ absorption maximum for all solvents, except R-13 methanol and chloroform residues, and the faint greenish fluorescence of these solutions suggests the presence of phytoene and phytofluene with the former absorbing at 265-295 mμ while the latter fluoresces bright green. This maximum was strongest for the yellow mutant ethanol residue which also showed an irregular absorption in the 760-356 mμ region. A weaker absorption maximum was recorded for the red mutant. This absorption maximum was generally weak for all residues, but definitely present for most samples. More work is needed to confirm the presence of these colorless, fluorescing carotenoids.

The acetone, benzene and methanol residues of the yellow

mutant gave irregular absorption curves generally from 760-590 mμ with the ethanol extending down to 356 mμ. The cause of this is unknown.

The special carotenoid test results showed that for both mutants the boiled water used to sacrifice the fish was yellow with bright green fluorescence for five of six fish and blue for one of six. Faint blue fluorescence was observed for three of five yellow mutants and two of five showed faint green-blue fluorescence. Permanganate-acetic acid media caused fluorescence to fade, results suggestive of pterins.

Acetone extracted golden yellow carotenoid(s) from the red mutants and yellow carotenoid(s) from the yellows. The difference in color intensities suggests a quantitative increase of this carotenoid in the red mutant.

For both mutants, thin layer chromatograms of acetone, petroleum ether and methanol extracts indicated no separation of materials; any carotenoids present were carried with the front.

Finally, the skin residue from the acetone extraction became intense red upon addition of 28% ammonia and fluoresced bright green (solution) and bright blue (tissues) for the red mutant. The same results were obtained for the yellows, with solution color and fluorescent intensity greatly reduced. Permanganate-acetic acid media caused fluorescence to fade. This indicated the presence of pterins.

Thin layer chromatograms of the fluorescent solutions gave

fluorescent spots as shown in Figure 20. For the red mutant, results closely resemble those of the red mutant pterin analysis. For the yellow, the chromatogram differs in that a green streak above the spot did not appear and the sample spot fluoresced blue. For both, the front stains brown with iodine vapor developer indicating an unseparated substance in the front (sepia- and/or isosepiapterin?).

Summarizing, the carotenoid data shows the following:

1. Fluorescing water soluble substances (pterins and/or flavins?) were dissolved from the slime layer or body tissues by boiling water with no further treatment.

2. Carotenoids were present in both mutants as evidenced by their solubility in organic solvents. A yellow to orange acetone soluble carotenoid was found in both mutants.

3. No evidence was found to indicate the presence of lutein or isolutein in either mutant though these were reported in Bettas by Goodrich, et al. (1941).

4. The red mutant had an orange carotenoid characterized by a single absorption maximum. It is thought to be astaxanthin. Yellow mutants lack this.

5. Colorless, fluorescing carotenoid(s) with absorption maximum at 278 mμ were found in both mutants but were more predominant in the yellows. Possibly this represents phytoene and/or phytofluene.

6. The sulfuric acid color test for carotenoids gave incon-

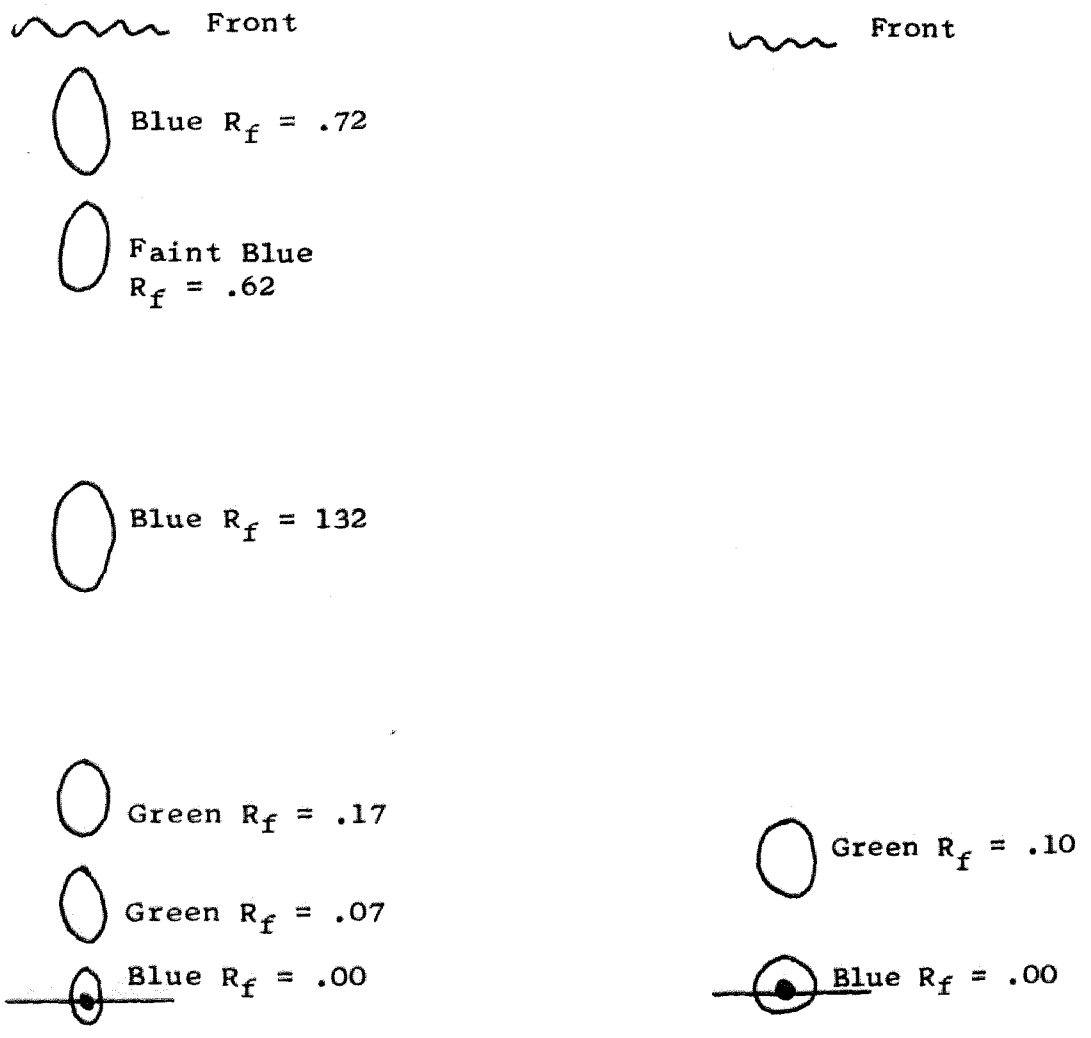


Figure 20. Pterin TLC results from red and yellow mutant skin residues after removal of carotenoids.

clusive results for both mutants.

7. After carotenoid extraction, skin residues fluoresced when treated with dilute ammonia. The ammonia solution gave TLC results similar to those obtained in pterin analysis.

Ziegler (1961) states that all naturally occurring pterins are derivatives of 2-amino-4-hydroxypteridine. Ziegler and others (Ziegler, 1961) found xanthopterin, a xanthopterin-like compound and biopterin to be degradation products of natural pterins; isoxanthopterin is an exception.

> "The natural products probably are the red pterin (consisting of three closely related pterins: drosopterin, isodrosopterin, and neodrosopterin ...), the yellow pterin (=sepiapterin ...) and a nonfluorescing pterin, which immediately starts to fluoresce after irradiation at 365 mμ .... This latter compound is a derivative of tetrahydrobiopterin .... It is known for certain that the yellow pterin is also a derivative of biopterin ...." (Ziegler, 1961)

Chromatographic studies of the yellow eyed Drosophila mutant, sepia, by Ziegler and Hadorn (Ziegler, 1961) showed a lack of red eye pigment coupled with an increase in xanthopterin, a xanthopterin-like pterin, yellow pterin (="sepia" pterin), 2-amino-4-hydroxypterin and biopterin. Refined, milder techniques later showed only a tetrahydrobiopterin derivative, indicating that

> "The increase in these three pterins found in earlier investigations ... is in reality only a reflection of an increase of the hydrogenated pterins (tetrahydrobiopterin derivative and yellow pterin) in the living tissues." (Ziegler, 1961)

Results of this experiment indicate both qualitative and

quantitative differences for red and yellow Betta mutants. Red drosopterins (=erythropterin? of Goodrich, et al., 1941) and astaxanthin color the red, while both are absent in the yellow. Quantitative increases in isoxantho- and/or xanthopterin occur in the yellows. Yellow mutants also lack 2-amino-4-hydroxypteridine and biopterin, both of which are present in reds. Summarizing:

RED	YELLOW
Drosopterins	"Modified" blue fluorescing substance (xanthopterin-like pterin?)
Isoxanthopterin	Isoxanthopterin
Xanthopterin	Xanthopterin
2-amino-4-hydroxypteridine	----
Biopterin	----
Sepiapterin	Sepiapterin
Isosepiapterin	Isosepiapterin

A pattern similar to that of the sepia mutant emerges for yellow Bettas. Red drosopterins are lacking with increases in isoxantho-, xanthoperin, a xanthopterin-like pterin, and yellow pterin. Milder (e.g., propanol:1% ammonia = 70:30) techniques would probably show only a tetrahydrobiopterin derivative present in the yellow mutant.

A second red pigmentary system appears in red-yellow mutant studies. Lutein, the xanthophyll reported by Goodrich, et al. (1941) was not found. The red-orange xanthophyll, astaxanthin,

is spectrophotometrically identified in red Bettas though absent in yellows. This suggests a second altered synthetic pathway. A detailed analysis is needed to determine if this Betta carotenoid originates as lutein and is then converted to astaxanthin.

Also unexplained is the suspected presence of the colorless fluorescing carotenoids, phytoene and phytofluene. They may be colorless intermediary products of astaxanthin synthesis, or they may occur independent of astaxanthin.

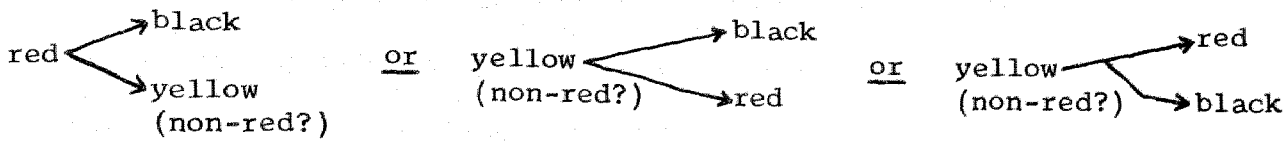
It appears that two red pigmentary systems operate in Bettas. Wild type Bettas do not show yellow and have red only on the fins. "Extended red" mutants show quantitative red pigment increase over the entire body and is dominant to "non-red" and wild type (normal red).

The "non-red" either shows yellow where red should be or lacks both. "Non-red" is independent of the cambodia mutation (an albinistic form). Cambodia may, however, inhibit red pigment on the body. "Non-red" is recessive to normal red.

Hypothetically, a biosynthetic pathway for Betta pigment production can by constructed as follows:

$$\underset{\text{(non-red?)}}{\text{colorless}} \xrightarrow[E_c]{} \underset{\text{(non-red?)}}{\text{yellow}} \xrightarrow[E_y]{} \text{red} \xrightarrow[E_r]{} \text{black}$$

A malfunction in production of one enzyme caused by a genetic mutation could result in a color variation. Alternate pathways include:



The first alternative seems highly unlikely since it implies both black and yellow pigmentation, where the black would mask the yellow. For the yellow to be apparent, black formation would have to be blocked as well as all red converted to yellow. In the second case, we again have the problem of masking by black. If, however, black were not produced, but red was, the red would be observable. The third alternative indicates synthesis of black as an alternate route to yellow to red (or red to yellow) pigment production.

Pigment cell deterioration and consequent color loss in Bettas may give visual clues to the correct synthetic route. As black pigments deteriorate, light colored irregular patches appear. According to Lucas (1968)

> "If pigment deterioration continues (and it usually does), iridocyte color and red soon follow. The process may be completed in a week or it may take several. It may be arrested or even reversed at any time. If color re-forms red develops first ... followed by iridocyte color, then melanin."

A red Betta may show color loss back to a colorless, or light yellow stage.

This series of color changes is most effectively explained by the second alternative for pterins [yellow → black / red] and the "colorless → yellow → red" route for carotenoids.

The observable flaw arising from selection of the first mechanism for pterins is the comparative rareness of black Bettas. Recall, however, that riboflavin and xanthopterin act antagonistically in melanogenesis. Xanthopterin inhibits melanogenesis, while riboflavin has the opposits effect. Many black Bettas show considerable yellow and/or red; they may also appear brownish rather than black. If the first synthetic route is correct, and if riboflavin is integral in melanogenesis, then red or yellow "black" Bettas probably result from a dietary lack of sufficient riboflavin, a hypothesis which should be experimentally tested.

Selection of the "yellow → black / red" pathway for pterins can explain color loss. Pterins have been shown to be related to melanin with both pigments present in the same chromatophores (Fox and Vevers, 1960). The black mutant probably is a phenotypic display of melanin while red mutants display pterins. Conceivably, both mutants arise from the "yellow," which possibly represents an intermediary in melanin and/or pterin synthesis. The melanin-producing enzyme system may be weakened because of low riboflavin and high xanthopterin concentrations. Melanogenesis may even by stopped at a "red" stage. Increased xanthopterin (melanogenesis inhibitor) would strengthen the pterin system thus producing "weak" black Bettas that easily undergo color loss, or, if the xanthopterin is "strong" enough, the mel-

anogenesis could be stopped and red pterins predominate.

This explanation is supported by experimental observation. A red substance is left in the tissues after dilute ammonia is added to remove pterins; melanins are unaffected by dilute acid or base, with concentrated base needed to dissolve them. Further research is needed to determine if this red substance is actually a form of melanin.

Further research is needed to confirm the "colorless ⟶ yellow ⟶ red" pathway for carotenoids and its relationship, if any, to pterinogenesis. Results indicate, however, that this system is operative in Bettas with the final red-orange product being astaxanthin. Blockage produces a colorless to yellow carotenoid in the yellow mutant.

If the two pigment systems are present and act independently, then four red-yellow mutants are possible:

1. "Red" - red pterins and carotenoids both present,
2. "Pterin-red" - red pterins and no red carotenoids,
3. "Carotenoid-red" - red carotenoids and no red pterins,
4. "Non-red" - no red pterins or carotenoids.

The "Red" should be the "heaviest" pigmented mutant. "Pterin-red" should be redder than "Carotenoid-red," the latter being an orange-red. "Non-red" produces yellow or colorless ("cellophane") Bettas.

Several questions arise from this research. Is the fluorescing material in the tissues actually pterins or could it be

flavins? Experimental evidence strongly suggests that the fluorescing materials in the tissues are pterins. Immediate diffusion into solution and chromatographic behavior support this. However, what substances are present in the slime layer over the tissues? Are flavins possible, and, if so, what effect do they have on the observations? Possibly extensions of chromatographic techniques used in slime layer analysis could be applied to this problem.

Possibly related to this problem is the blue fluorescence displayed by the boiled water used to sacrifice the fish. The boiling water would break some of the pterin-protein bonds freeing fluorescing pterins. The same can be said of flavins. Further work is needed to determine if this fluorescing substance is pterins, flavins or something else, and if its origin is the slime layer or body tissues.

SUMMARY

An attempt was made to obtain evidence that yellow Betta splendens are mutants of red. Pigment extractions were made and pigments analysed by physical and chemical means. Tentative identifications were made based on comparisons with previously identified substances in other organisms.

Red mutants have both pterin and carotenoid pigments. Red drosopterins and red-orange carotenoid (astaxanthin) color this

mutant. Other fluorescing pterins are present. These include isoxantho- and xanthopterin, 2-amino-4-hydroxypteridine, biopterin and probably sepia- and isosepiapterin (=yellow pterins). Colorless fluorescing carotenoids, thought to be phytoene and phytofluene, were also present.

Yellow mutnats have both pterin and carotenoid pigments, but not the same ones as the reds. The yellows lack drosopterins and the red-orange carotenoid (astaxanthin). The red pterin appears to be altered to a blue fluorescing, "colorless" pterin in the yellow. Isoxantho- and xanthopterin are present, and, probably, sepia- and isosepiapterin (=yellow pterins). The colorless fluorescing carotenoids were present in increased amounts in the yellows.

Possible synthetic pathways were considered based again upon comparisons with those worked out for other organisms. It appears that two pigmentary systems produce red Betta color. Drosopterins are the natural end product of the pterin system. If a break in the synthesis occurs, tetrahydrobiopterin derivatives are not converted to red and a yellow or colorless phenotype results. The carotenoid system appears to form red-orange astaxanthin as an end product. Incomplete synthesis also results in a yellow or colorless phenotype.

This double system indicates four possible phenotypes. "Red" has both red pterins and carotenoids, "Pterin-red" has only pterin red (drosopterins) and no red carotenoid, "Carotenoid-

red" has only red carotenoid (astaxanthin) and no red pterins, and "Non-red" lacks both red pterins and carotenoids.

Additional studies are needed to definitely identify the red-orange carotenoid and the colorless, fluorescing carotenoids. Milder pterin chromatography is needed to show that the various fluorescing pterins of the yellow mutant are actually colorless, fluorescing tetrahydrobiopterin derivatives indicative of interrupted drosopterins synthesis.

Genetic information is needed to confirm the presence of two red pigment forming systems in Bettas. Crosses should be made to determine if these types of red can be segregated, if and how they are related, and if and how they might be dependent on other factors.

Finally, the fluorescing hot water soluble material needs to be identified. It appears to be pterins, but could also be a flavin or the water soluble fluorescing carotenoid, fluorescyanin. It should also be determined whether this material arises from the slime layer or the body tissues of the fish.

LITERATURE CITED

Bobbitt, James M., Arthur E. Schwarting and Roy J. Gritter. 1968. Introduction to Chromatography. Reinhold Book Corp.: New York.

Brooks, E. W. 1955. The Development of Color in Budgerigars. All-Pets Books, Inc.: Fond du Lac, Wisconsin.

Cantarow, Abraham and Bernard Schepartz. 1963. Biochemistry. 3rd ed. W. B. Saunders Co.: Philadelphia.

Foster, Morris. 1965. "Mammalian Pigment Genetics." Acvance. in Genet. 13:311-339. Academic Press: New York.

Fox, Denis L. 1953. Animal Biochromes and Structural Colours. Cambridge University Press: Cambridge, England.

Fox, H. Munro and Gwynne Vevers. 1960. The Nature of Animal Colours. Sidgwick & Jackson Limited: London.

Gelman Instrument Co. 1968. Principles and Procedures of Instant Thin Layer Chromatography. Product Bulletin 287B. Ann Arbor, Michigan.

Goodrich, H. B., C. A. Hill and Myron S. Arrick. 1941. "The Chemical Identification of Gene-Controlled Pigments in Platypolcilus and Xiphophorus and Comparisons with Other Tropical Fish." Genetics 26:571-585. Academic Press: New York.

Goodrich, H. B. and R. N. Mercer. 1934. "Genetics and Colors of the Siamese Fighting fish." Science 79:318-319. Amer. Ass. for the Advance. of Sci.: Washington, D.C.

Goodwin, T. W. 1955. "Carotenoids." Modern Methods of Plant Analysis. Vol. III. Springer-Verlag: Berlin.

Goodwin, T. W. 1964. "The Chemistry of Animal Colours." Colour and Life. Symposia of the Institute of Biology No. 12. The Institute of Biology: London.

Hadorn, Ernest. 1962. "Fractionating the Fruit Fly." Sci. Amer. 206 no. 4:100-113. Scientific American, Inc.: New York.

Hawks, Philip B., Bernard L. Oster and Wm. H. Summerson. 1947. Practical Physiological Chemistry. The Blakiston Co.: Philadelphia.

Lucas, Gene A. 1968. A Study of Variations in the Siamese Fighting Fish, Betta splendens, with Emphasis on Color Mutants and the Problem of Sex Determination. Unpublished Ph.D. Thesis. Iowa State University: Ames, Iowa.

Mertens, Thomas R. and Alice S. Bennett. 1969. "Drosophila Chromatography-Again." Turtox News 47 no. 6:220-221. CCM: General Biological, Inc.: Chicago.

Mohrig, Jerry R. and Douglas C. Neckers. 1968. Laboratory Experiments in Organic Chemistry. Reinhold Book Corp.: New York.

Morrison, Robert T. and Robert N. Boyd. 1967. Organic Chemistry. 2nd ed. Allyn and Bacon, Inc.: Boston.

Silverstein, Robert M. and G. Clayton Bassler. 1963. Spectrometric Identification of Organic Compounds. John Wiley and Sons: New York.

Stein, Wm. H. and Stanford Moore. 1951. "Chromatography." Sci. Amer. reprint #81. W. H. Freeman & Co.: San Francisco, California.

Stock, R. and C. B. F. Rice. 1967. Chromatographic Methods. 2nd ed. Chapman & Hall Ltd.: London.

Wallbrunn, Henry M. 1951. Genetics of the Siamese Fighting Fish, Betta splendens. Xeroxed copy, Unpublished Ph.D. Thesis; Chicago, Ill., Library, University of Chicago. University Microfilm, Inc.

Willard, Hobart H., Lynne L. Merritt, Jr. and John A. Dean. 1965. Instrumental Methods of Analysis. 4th ed. D. Van Nostrand Co., Inc.: Princeton, New Jersey.

Ziegler, Irmgard. 1961. "Genetic Aspects of Ommochrome and Pterin Pigments." Advance. in Genet. 10:349-395. Academic Press: New York.